University of Alberta

Oral Delivery of Insulin by Polymer-Coated Liposomes

by

Majid Tabbakhian ©

A Thesis

Submitted to the Faculty of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in

Pharmaceutical Sciences (Pharmaceutics)

Faculty of Pharmacy and Pharmaceutical Sciences
Edmonton, Alberta

Spring 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-29117-0

Canada

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Oral Delivery of Insulin by Polymer-Coated Liposomes** submitted by **Majid Tabbakhian** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Pharmaceutical Sciences (Pharmaceutics).**

Dr. J. A. Rogers (Supervisor)

Dr. Y. K. Tam

Dr. L. I. Wiebe

Dr. F. Pasutto

Dr. F. F. Cantwell

Dr. M. B. Bally (external examiner)

Date: Feb 27th, 1998

Dedicated to:

My beloved parents who raised me with love and affection, and taught me truth, honesty and the true meaning of generosity. Words can not convey my deepest feelings of appreciation and gratitude to them. May God bless them with peace and joy.

My dear sisters who will always hold a very special place in my heart.

And finally, my beloved wife whose kindness, love and encouragement were essential in the completion of my work. I am forever indebted to her for her endless patience and support.

ABSTRACT

The aim of this research was to formulate liposomes for oral delivery of insulin as a model protein drug. Studies were undertaken to establish optimal liposome compositions and preparation method to yield high entrapment of insulin, to determine the impact of cholesterol-derivatized mannan (CHM) and carboxymethyl chitin (CM-chitin) to reduce leakage in vitro, and to examine the performance of coated and uncoated liposomes administered in vivo to rats. A modified gradient HPLC technique was used to analyze insulin after solubilizing sample liposomes with 20 mM cetyltrimethylammonium bromide. The encapsulation efficiency (EE) of insulin reached 67 and 22 percent in positively- and negatively-charged liposomes, respectively, by the classic film method but increased to 46 percent in negatively-charged liposomes prepared by the dehydration-rehydration method. CHM increased EE whereas CM-chitin-coated liposomes had lower EEs correlating with their coating efficiencies. The amount of insulin released (leaked) was approximately 25 percent after 4 h from coated or uncoated liposomes in simulated gastric fluid (SGF) at pH 2.0 and 37°C. In comparison, the amount released in simulated intestinal fluid (SIF) at pH 7.4 was decreased by 50 percent after coating negatively-charged liposomes with CHM or by 32 percent from coated positively-charged liposomes. CM-chitin produced no significant improvement in leakage of insulin. The interaction of polymer with bilayers was elucidated using differential scanning calorimetry (DSC).

The intragastric administration of 400 U/kg insulin solution to diabetic rats did not lower blood glucose levels whereas 35 U/kg insulin in CHM-coated negatively-charged liposomes significantly decreased levels over a 2 h-period. A linear relationship between

areas above plasma glucose concentration-time curves ($AAC_{0\text{-}12\ h}$) and log dose was found ($r^2 > 0.95$).

This research has demonstrated the possibility of encapsulating insulin in liposome compositions that provide low leakage under simulated conditions of oral administration. The anchoring of CHM to the liposome bilayers appears to be essential since CM-chitin-coated liposomes had little stabilizing effect. Administered orally, CHM-coated liposomes of insulin exerted a significant hypoglycemic effect compared to uncoated liposomes or free insulin in diabetic rats. It is concluded that coating liposomes with CHM enabled more insulin to be absorbed from the GI tract than would otherwise be the case.

ACKNOWLEDGMENTS

I am grateful to Dr. James A. Rogers for his excellent supervision, invaluable advice, and great patience throughout the course of the program.

Sincere thanks are extended by the author to Dr. Yun K. Tam, Dr. Leonard Wiebe, and Dr. David F. Biggs for their invaluable recommendations, suggestions, enthusiasm, and motivation, and to Dr. Marcel M. Bally for his critical evaluation and invaluable comments on this thesis. I would also like to acknowledge Dr. Glen Kwon, Dr. Franco M. Pasutto, and Dr. F. F. Cantwell for their valuable input.

The author would like to truly thank Dr. K. Parang for his advice on polymer derivatization and Dr. R. V. Rajotte for his guidance and assistance in diabetes studies.

The assistance of Dr. T. M. Allen's lab for the particle size measurements is appreciated.

I wish to genuinely thank Mr. Keith E. Anderson for his help and contribution to scientific discussions on this study. Technical assistance from Mr. Don Whyte and Dr. Somayji is greatly appreciated.

Financial support for this project and the author was provided by the Ministry of Heath of Iran, Medical Research Council of Canada, and Faculty of Pharmacy, University of Alberta.

TABLE OF CONTENTS

1. INTRODUCTION 1

1.1. OVERVIEW 1

1.2. BACKGROUND 2

1.2.1. Biological Barriers to Oral Peptide and Protein Absorption 3

1.2.1.1. Enzymatic barrier 3

1.2.1.2. Physical barrier 4

1.2.1.2.1. Peptide and protein transport across the GI tract 7

1.2.1.2.2. Paracellular transport 8

1.2.2. Approaches to Maximize the Oral Absorption of Protein Drugs 10

1.2.2.1. Chemical modification of drugs 10

1.2.2.2. Co-administration with protease inhibitors 11

1.2.2.3. Use of penetration enhancers 13

1.2.2.4. Formulation and drug delivery approaches 15

1.2.2.4.1. Small intestine 15

1.2.2.4.2. Colon 16

1.2.2.4.3. Peyer's patches 17

1.2.2.4.4. Lymphatic system 19

1.2.3. The Liposome as a Drug Delivery System 20

1.2.3.1. Properties/characteristics of liposomes 21

1.2.3.1.1. Composition 21

1.2.3.1.2. Phase behavior 23

1.2.3.1.3. Bilayer permeability 24

1.2.3.1.4. Size 25

1.2.3.2. In vivo behavior of liposomes 26

1.2.3.2.1. Polymerized liposomes 27

1.2.3.2.2. Surface-modified liposomes 28

1.2.3.3. Liposomal formulation of protein/peptide drugs 32

1.2.3.3.1. Peptide/protein drug properties considerations 33

1.2.3.3.2. Liposome preparation techniques 33

1.2.4 Insulin 36

1.2.4.1. Pharmacology 36

1.2.4.2. Physicochemical properties 37

1.2.4.3. Assay 38

1.2.4.4. Route of administratiom 38

1.2.5. Oral Administration of Liposomes 39

1.3. RATIONALE 41

1.4. AIM 41

1.5. HYPOTHESES 41

1.6. OBJECTIVES 42

2.. EXPERIMENTAL 44

2.1. MATERIALS 44

2.2. METHODS 44

2.2.1. Polymer Derivatization 44

2.2.2. Analytical 50

2.2.2.1. HPLC analysis of insulin 50

2.2.2.2. Determination of insulin adsorption 50

2.2.3. Liposome Preparation 51

2.2.3.1. Classic film method 51

2.2.3.2. Dehydration-rehydration (DRV) method 52

2.2.4. Liposome Characterization 53

2.2.4.1. Determination of polymer coating efficiency 53

2.2.4.2. Encapsulation efficiency 53

2.2.4.3. Particle size measurement 54

2.2.4.4. Differential scanning calorimetry (DSC) 54

2.2.5. Liposome Behavior in Various Media 55

2.2.5.1. Stability in sodium cholate solutions 55

2.2.5.2. Stability of insulin in liposomes in α-chymotrypsin solutions 55

2.2.5.3. Release of insulin 56

2.2.5.3.1. In buffer solution 56

2.2.5.3.2. In simulated gastric and intestinal fluids 57

2.2.6. In Vivo Studies 57

2.2.6.1. Animals 57

2.2.6.2. Induction of diabetes mellitus 58

2.2.6.3. Dosing and sampling 58

2.2.6.4. Glucose assay 59

2.2.6.5. Treatment of data 59

2.2.6.6. Pharmacodynamic analysis 59

3. RESULTS 60

3.1. POLYMER DERIVATIZATION 60

3.2. ANALYTICAL 63

3.2.1. Insulin Assay 63

3.2.2. Insulin Adsorption 63

3.3. LIPOSOME CHARACTERIZATION 67

3.3.1. Coating Efficiency of Liposomes by CM-chitin and CHM 67

3.3.2. Insulin Encapsulation Efficiency (EE) and Loading Efficiency (LE) 70

3.3.3. Liposome Size Determination 76

3.3.4. DSC Study of liposomes 81

3.4. LIPOSOME BEHAVIOR IN VARIOUS MEDIA 85

3.4.1. Stability of liposomes in bile salt solutions 85

3.4.2. Stability of insulin in liposomes in the presence of enzymes 85

3.4.3. Insulin Release 86

3.5. IN VIVO STUDIES 102

4. DISCUSSION 113

4.1. POLYMER DERIVATIZATION 113

4.2. ANALYTICAL 117

4.3. LIPOSOME CHARACTERIZATION 119

4.3.1. Coating Efficiency (CE) 119

4.3.2. Encapsulation Efficiency (EE) 120

4.3.3. Size Study 126

4.3.4. Differential Scanning Calorimetric Studies of Liposomes 127

4.4. LIPOSOME BEHAVIOR IN VARIOUS MEDIA 132

4.4.1. Stability in bile salt solutions 132

4.4.2. Stability of insulin in liposomes in the presence of enzymes 133

4.4.3. Release of insulin 134

4.5. IN VIVO STUDIES 135

5. SUMMARY 139

6. CONCLUSIONS 143

7. FUTURE WORK 145

REFERENCES 146

LIST OF TABLES

Table 2.1. Elemental analysis following ethylendiamine substitution of carboxymethylated polysaccharides 48

Table 3.1. Summary of polysaccharides derivatization details 62

Table 3.2. Summary of insulin EE and loading in PCL and NCL as functions of preparation techniques and polymer coating[a,b] 73

Table 3.3. Liposome size as a function of preparation technique and polymer coating [a,b] 80

Table 3.4. Thermodynamic characteristics of DPPC liposomes[a] 84

Table 4.1. Comparison of insulin encapsulated or associated with the outer layer of preformed empty PCL (DPPC:CH:SA, 7:2:1 m.r.) prepared by the CF method[a]. 125

Table 4.2. Comparison of insulin EE (%) in DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes prepared by the DRV method where insulin solution was introduced to empty SUVs before or after freeze-drying[a] 125

LIST OF FIGURES

Figure 1.1. Schematic representations of (a) two villi and a crypt and (b) the organization of the organization of the intestinal epithelial absorptive cells (from reference 16). 5

Figure 1.2. Possible approaches to stabilize liposomes via surface coating with polymers (from reference 146). 30

Figure 2.2. Mannan and its derivatives 48

Figure 2.1. A typical IR spectrum of carboxymethyl polysaccharide (e.g. CM-mannan) in KBr. The presence of a carbonyl peak at 1741 cm^{-1} is evidence of carboxymethylation. 47

Figure 3.1. ^{1}H-NMR spectra of CHM in DMSO-d_6 at RT. Inset (a) and (b) are mannan and cholesterol spectra, respectively. Protons of mannan from CHM appeared at 2.6-6.6 ppm and of cholesterol at 0.2-2.4 ppm as evidence of derivatization.. 61

Figure 3.2. HPLC chromatograms of (a) phenol, (b) insulin, (c) desamido-insulin obtained by a gradient control method consisting of mobile phase A, 0.055% trifluoroacetic acid (TFA) in water, and mobile phase B, 0.050% TFA in acetonitrile. A linear gradient was run from 25 to 40 percent mobile phase B within 15 min, maintained at 40 percent mobile phase B for 8 min, then

returned to the initial concentration (25/75, B/A ratio) and maintained for 15 min prior to the next injection. Flow rate, constant at 1 ml/min; detection, UV at 210 nm; column, C18 μBondapack (Millipore®). 64

Figure 3.3. A typical standard curve of insulin determined by a gradient control HPLC method (see details in Fig 3.2), n=3, $r^2 > 0.99$. 65

Figure 3.4. Comparative adsorption of insulin on various tube surfaces after 1h incubation at RT. ▭, polypropylene; ▨, uncoated glass; ▩, dimethyldichlorosilane-; and ■, Surfasil-coated glass tubes. The peak areas of insulin at each concentration were normalized based on the value obtained from Surfasil-coated glass tubes which generally gave the highest responses 66

Figure 3.5. Turbidity standard curves of CHM (upper) and CM-chitin (lower) dispersions in a 20 mM CTAB (pH 7.4):isopropanol (9:1 v/v) solution determined at 400 nm after (●) 15 min, (O) 60 min and (▼) 16 h incubation. Values represent means of 2 or 3. 68

Figure 3.6. Coating efficiency of (a) DPPC:CH:SA (7:2:1 m.r.) liposomes, and (b) DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes as a function of polymer concentration at pH 7.4. Coating efficiency is

defined as mg polymer associated with 100 mg lipid. Means ± SE, n=3. 69

Figure 3.7. The effects of detergents on the assessment of EE of insulin in DPPC:CH:SA (7:2:X m.r.) liposomes. Insulin was measured by HPLC following liposome disruption in a solution of 5% SDS, ■; or 20 mM CTAB, ▭ Final lipid concentrations were 0.5 mM. Liposomes treated with detergents were heated > T_m of DPPC for 5 min. Means ± SE, n=3 71

Figure 3.8. EE of insulin by DPPC:CH:DMPG (7:2:Y m.r.) liposomes prepared by the CF method following pellet disruption by 20 mM CTAB. Final lipid concentrations were 0.5 mM. Liposomes treated with detergents were heated above T_m of DPPC for 5 min. Means ± SE, n=3. 72

Figure 3.9. Effect of the volume of insulin solution on insulin EE in NCL of various compositions prepared by the CF method. All solutions contained the same amount of insulin. ▭, DPPC:CH:DPPG (7:1:0.5 m.r.); ▨, DPPC:CH:DPPG (7:2:0.5 m.r.); ▨, DOPC:DPPG (7:0.5 m.r.). Means ± SE (n=3) 73

Figure 3.10. Effects of CHM-coating on insulin EE in NCL composed of DPPC:CH:DMPG (7:2:0.5 m.r.) prepared by the CF method. Means ± SE, n=3. 75

Figure 3.11. Log-probability plots of sizes of liposomes composed of DPPC:CH:DPPG (7:2:X m.r.) measured by dynamic laser light scattering (Brookhaven Instruments BI-90 Particle Sizer, Holtsville, New York), where x denotes mole ratio of DPPG as ●, 2; ■,1; ▼, 0.5; and ◆, 0.25. 77

Figure 3.12. An example of log-probability plots of sizes of liposomes composed of DPPC:CH:DMPG (7:2:X m.r.) measured by dynamic laser light scattering (Brookhaven Instruments BI-90 Particle Sizer), where x denotes mole ratio of DMPG as ●, 2; ■,1; and ▼, 0.5..... 78

Figure 3.13. Effect of SA mole ratio on the size of DPPC:CH(7:2 m.r.) liposomes prepared by the CF method. Means ± SE, n=3..... 79

Figure 3.14. Effect of mole ratio of charged adduct on the size of DPPC:CH liposomes (7:2 m.r.) prepared by the CF method, ●, DMPG; ■, DPPG. Means ±SE, n=3. 79

Figure 3.15. Effects of CH and charged adducts (SA or DMPG) on DSC thermograms of DPPC liposomes. Inset (b) shows enlargement of the thermogram of DPPC:CH (7:2 m.r.) liposomes from (a)..... 82

Figure 3.16. Effects of insulin and polymer coating on the DSC thermograms of, (a) neutral DPPC liposomes, (b) positively-charged

DPPC:SA (7:1 m.r.) liposomes (PCL), and (c) negatively-charged DPPC:DMPG (7:0.5 m.r.) liposomes (NCL). 83

Figure 3.17. An absorbance standard curve of DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes as a function of lipid concentration at 400 nm and RT. 87

Figure 3.18. Relative stability of DPPC:CH:SA (7:2:1 m.r.) liposomes as a function of SC concentration at pH 7.4 and RT; (a) after 15 min, and (b) after 60 min incubation. ■, CHM-coated; ●, CM-chitin-coated; and ◆, uncoated liposomes. Mean ± SE, n=3. 88

Figure 3.19. Relative stability of DPPC:CH:SA (7:2:1 m.r.) liposomes as a function of SC concentration after 15 min incubation at pH 7.4 and 37°C. ■, CHM-coated; ●, CM-chitin-coated; and ◆, uncoated liposomes. Means ± SE, n=3. 89

Figure 3.20. Relative stability of DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes as a function of SC concentration at pH 7.4 and RT. (a) after 15 min, and (b) after 60 min incubation. ■, CHM-coated; ●, empty liposomes; and ▽, uncoated liposomes, Means ± SE, n=3. 90

Figure 3.21. Stability of insulin solution or insulin encapsulated in uncoated- or CHM-coated- NCL (DPPC:CH:DMPG, 7:2:0.5 m.r.) in the presence of pepsin or α-chymotrypsin at 37 °C after 0.5 h, ▭; 2 h, ▨; or 4 h, ▩ incubation period. Means ± SE, n=3. 91

Figure 3.22. Insulin release from DPPC:CH:SA (7:2:1 m.r.) liposomes in SGF at 37 °C. ●, uncoated and ▼, CM-chitin-coated. 92

Figure 3.23. Comparison of insulin release from CM-chitin-PCL in PBS (▽) and in SIF (▼) at 37 °C. Means ± SE, n=3. 93

Figure 3.24. ^{125}I-insulin release from DPPC:CH:SA (7:2:1 m.r.) liposomes in SIF at 37 °C. ●, uncoated, ■, CHM-coated, and ▼, CM-chitin-coated. 94

Figure 3.25. ^{125}I-insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SGF at 37 °C. ●, uncoated; ■, CHM-coated; and ▼, CM-chitin-coated. 95

Figure 3.26. Comparison of insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SGF (closed symbols) or SGF without enzymes (open symbols) at 37 °C. Uncoated (circles); CHM-coated (squares). 96

Figure 3.27. ^{125}I-insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SIF at 37 °C. ●, uncoated; ■, CHM-coated; ▼, CM-chitin-coated. 97

Figure 3.28. Comparison of insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SIF (closed symbols) or SIF without enzymes

(open symbols) at 37 °C. Circles, uncoated; and squares, CHM-coated..... 98

Figure 3.29. Release of insulin in SGF without enzymes at 37 °C as a function of liposome composition and polymer coating. Open symbols, DPPC:CH:DMPG (7:2:0.5 m.r.); open symbols with plus signs, DSPC:CH:DMPG (7:2:0.5 m.r.). Circles, uncoated, and squares, CHM-coated..... 99

Figure 3.30. Release of insulin in SIF without enzymes at 37 °C as a function of liposome composition and polymer coating. Open symbols, DPPC:CH:DMPG (7:2:0.5 m.r.); open symbols with plus signs, DSPC:CH:DMPG (7:2:0.5 m.r.). Circles, uncoated; squares, CHM-coated. 100

Figure 3.31. Effect of the method of treatment of liposomal insulin on the assessment of drug release. Comparison of insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SGF at 37 °C. Closed symbols, direct determination of release of ^{125}I-insulin by γ-counting; open symbols, determination of insulin in liposomal pellet after deactivation of pepsin in the medium. Circles, uncoated; squares, CHM-coated..... 101

Figure 3.32. The calibration curve of glucose measured by a Beckman® Astra-8® analyzer (■) or a Medisense® blood glucose sensor

electrode apparatus (●). Units of mmol/l obtained by the Medisense® apparatus were converted to mg/dl by multiplying by 18.02. 104

Figure 3.33. Typical blood glucose levels of rats with (●) or without diabetes (○). Diabetes was induced by i.v. injection of 55 mg/kg streptozocin (STZ) via the pineal vein and blood glucose sampling occurred on days 7, 8, and 9 after STZ injection. Means ± SE, n = 3. 105

Figure 3.34 Blood glucose levels (%) in fasted diabetic rats (control groups) following intragastric administration of (⊙), 400 U/kg of insulin solution, (□), pH 7.4 PBS solution, (○), empty liposomes, or (▽) s.c. injection of 2 U/kg insulin. Means ± SE, n = 6 or 8..... 106

Figure 3.35. Comparison of the hypoglycemic effects of insulin-containing uncoated or CHM-coated negatively-charged liposomes (CHM-NCL) following intragastric administration of a formulation to fasted diabetic rats. (□), 280 U/kg insulin in U-NCL; (■), 280 U/kg insulin in CHM-NCL; (⊙), 400 U/kg insulin solution (as control group). Means ± SE, n = 6 - 8; *, $P < 0.05$..... 107

Figure 3.36. Comparison of the hypoglycemic effects of insulin containing uncoated or CHM-PCL following intragastric administration to

fasted diabetic rats. (◇), 280 U/kg insulin in U-PCL; (◆), 280 U/kg insulin in CHM-PCL; (⊙), 400 U/kg insulin solution (as control). Means ± SE, n = 6 - 8; *, P < 0.05 108

Figure 3.37. Comparison of the hypoglycemic effects of insulin liposomes following intragastric administration to fasted diabetic rats as a function of liposome charge. (a) uncoated, and (b) CHM-coated. (◇), 280 U/kg insulin, U-PCL, (◆), 280 U/kg insulin, CHM-PCL, (□), 280 U/kg insulin, U-NCL, (■), 280 U/kg CHM-NCL, and (⊙), 400 U/kg insulin, solution (as control). Curves were extracted from figures 3.35 and 3.36 and rearranged. Mean ± SE, n = 6 - 8, *, P < 0.05 109

Figure 3.38. Blood glucose levels (%) following intragastric administration of various doses of insulin as CHM-NCL to fasted diabetic rats. (▼), 35 U/kg; (●) 70 U/kg; (■), 280 U/kg; (▲), 400 U/kg; (⊙), 400 U/kg of insulin solution (as control). Means ± SE, n = 6 - 8, *, P < 0.05 110

Figure 3.39. Dose-effect relationship of insulin encapsulated in CHM-NCL formulations intragastrically administered to fasted diabetic rats ($r^2 > 0.95$)..... 111

Figure 3.40. Comparison of the area above the plasma glucose concentration-time curves (%blood glucose.h), $AAC_{0\text{-}12h}$, of intragastrically-

administered formulations in fasted diabetic rats. The base line was the mean of blood glucose concentrations prior to administration of formulations. CHM-, cholesteryl-mannan; U, uncoated; NCL, negatively-charged liposomes consisted of DSPC:CH:DMPG (7:2:0.5 m.r.) prepared by DRV method; PCL, positively-charged liposomes consisted of DSPC:CH:SA (7:2:1 m.r.) prepared by DRV method; PBS, pH 7.4 phosphate buffer saline solution; Insulin, insulin solution; Insulin s.c., subcutaneously injected insulin; and EL, empty liposomes. The number after each abbreviation represents the insulin dose administered..... 112

Figure 4.1. The modified carboxymethylation steps of polysaccharides (e.g. mannan) derivatization (a-c) compared to single derivatization step of Inman [164,165,205], step d. 115

Figure 4.2. Ethylenediamine (a) and cholesterol (b) derivatization of carboxymethyl polysaccharides (e.g. mannan) as described in references 164,165,205, yielding the final cholesterol aminoethylcarbomethyl (AECM) polysaccharide, e.g. CHM..... 116

Figure 4.3. Effects of CM-chitin and CTAB on the peak area of insulin resolved from HPLC. Means ± SE (n=3), *, $P < 0.05$..... 119

Figure 4.4. Comparison of insulin EE in uncoated and CHM-coated-PCL and NCL prepared by the DRV method. Means ± SE, n=3 (values extracted from Table 3.2). 124

Figure 4.5 The effect of insulin incorporation or polymer-coating on the enthalpy change (ΔH°) of the main transition endotherms of neutral liposomes (NL), PCL, and NCL composed of DPPC, DPPC:SA (7:1 m.r.), and DPPC:DMPG (7:0.5 m.r.) respectively. For the sake of comparison, the enthalpy changes of the transitions of all systems were subtracted from that of liposomes composed of DPPC only (horizontal line at zero). Values obtained from Table 3.4. ▭, empty and uncoated; ▨, insulin-encapsulated and uncoated; ▩, CM-chitin-coated; and ▩, CHM-coated. 131

Figure 5.1. Optimization of the EE of insulin (square) and the size (circle) of (a) DPPC:CH:SA (7:2:X m.r.) or (b) DPPC:CH:DMPG (7:2:Y m.r.) liposomes prepared by the CF method as a function of SA or DPMG (closed symbols) or polymer concentration (open symbols). Notes: 1) Effects of polymer concentrations were evaluated at fixed SA or DMPG mole ratios of 1 and 0.5, respectively. 2) Plots are extracted from **RESULTS** and re-arranged. 140

LIST OF ABBREVIATIONS

AAC	Area above (the plasma glucose) concentration-time curve
AECM-	Aminoethylcarbomethyl
ANOVA	Analysis of variance
Ara-C	Cytarabine arabinoside (cytarabine)
BSA	Bovine serum albumin
CE	Coating efficiency
CF	Classic film method (of liposome preparation)
CH	Cholesterol
CHD	Cholesterol-derivatized dextran
CHM	Cholesterol-derivatized mannan
CHP	Cholesterol-derivatized pullulan
CM-	Carboxymethyl
CM-chitin	Carboxymethyl chitin
CTAB	Cetyltrimethylammonium bromide
DDS	Drug delivery system
DMF	Dimethylformamide
DMPC	Dimyristoylphosphatidylcholine

DMPG	Dimyristoylphosphatidylglycerol
DMSO	Dimethylsulfoxide
DOPC	Dioleoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DPPG	Dipalmitoylphosphatidylglycerol
DRVs	Dehydration-rehydration vesicles
DSC	Differential scanning calorimetry
DSPC	Distearylphosphatidylcholine
EE	Encapsulation efficiency
FATMLVs	Freeze and thaw multilamellar vesicles
GI	Gastrointestinal
i.v.	Intravenous
LUVs	Large unilamellar vesicles
M-cells	Microfold cells
MLVs	Multilamellar vesicles
MPS	Mononuclear phagocytic system
m.r.	Mole ratio
NCL	Negatively-charged liposomes
NL	Neutral liposomes

PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCL	Positively-charged liposomes
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Polysaccharide
REVs	Reverse evaporation vesicles
RT	Room temperature
SA	Stearylamine
s.c.	Subcutaneous
SC	Sodium cholate
SGF	Simulated gastric fluid, USP
SIF	Simulated intestinal fluid, USP
SUVs	Small unilamellar vesicles
T_m	Phase transition temperature
U	Unit (of activity)

1. INTRODUCTION

1.1. Overview

Peptide and protein drugs have been of great interest in recent years because of the significance and remarkable scope of their functions and the diversity of their roles in biological processes [1]. While there have been dramatic advances in large-scale manufacturing along with a rapid progress in pharmacology of peptides and proteins, progress in formulation and development has lagged behind [1,2]. This is largely due to the unique requirements and restrictions that peptides pose to the formulation chemist compared to conventional drug compounds. These properties include large molecular size, susceptibility to proteolysis, rapid plasma clearance, sometimes an unusual dose-response curve, immunogenicity, and a tendency to undergo aggregation, adsorption, and denaturation [3].

Demands for effective peptide/protein drug delivery systems has created innovation in the scope and complexity of drug delivery technology. Most protein-based therapeutic agents are still administered by parenteral routes which is inconvenient to patients and health professionals. Alternatively, other formulations such as controlled-release implants, implantable pumps, polymeric microspheres, liposomes, and peptide/protein conjugates are also used [1]. Other routes of administration are also being explored such as oral, nasal, pulmonary, rectal, vaginal, transdermal and ocular but difficulties have slowed progress [4,5]. The oral route generally is the most convenient and acceptable way

of drug delivery to patients especially for long term treatment of diseases. Hence, the development of an oral dosage form providing adequate bioavailability of insulin is appealing. Although it may not be used as an entire replacement of parenteral therapy, oral administration may be able to supplement deficient levels of endogenous insulin [6].

1.2. Background

The aim of this section is: (1) to review the nature of biological barriers to oral absorption of peptides, (2) to review the approaches to overcome these barriers and possibly maximize the oral bioavailability of peptide drugs, and (3) to provide an overview of the properties and characteristics of liposomes and polymer-coated liposomes and their potential as an oral delivery system for peptide and protein drugs.

The oral route is traditionally believed to be the least likely route for delivery of peptides and proteins, due to rapid and extensive degradation of the drug in the gastrointestinal (GI) tract, low permeability of large molecules across enterocytes, and post-absorptive first-pass hepatic clearance. Thus, if any success is to be achieved at all the protein or peptide must be engineered in form(s) that will offer protection and at the very least facilitate absorption of therapeutic amounts in the GI tract. Formulation as a microparticulate system enables a larger surface area exposure of the dosage form to absorbing sites and, presumably better absorption. Thus, formulation of peptides/proteins as microparticulates, and particularly liposomes, for oral administration and the barriers which they must overcome will be addressed.

1.2.1. Biological Barriers to Oral Peptide and Protein Absorption

1.2.1.1. Enzymatic barrier

Peptides and proteins are metabolized by numerous proteases or enzyme systems throughout the body. Metabolism can occur in two forms: (1) hydrolysis by proteases, such as rennin, enkephalinases and (2) chemical transformation, such as oxidation by oxidases and phosphorylation by kinases. Proteolysis is by far the more common [7].

Most proteases are present in a wide range of cell types in a variety of different organs. Thus, together these enzymes form an extremely effective barrier, preventing the access of a wide range of peptides to the underlying tissues. On the other hand, a peptide substrate is likely to be acted on by several of the enzymes either simultaneously or in quick succession. This usually happens in multiple anatomical sites, including the site of administration, the blood, the liver, the kidney, and the vascular endothelia [8,9]. The other feature of the enzymatic barrier is that almost all of the proteases capable of metabolizing a given peptide or protein are likely to be present in a given anatomical site in which the peptide or protein is located [10-12].

The implications are that, in order to successfully deliver the peptide and protein drugs, they must be protected against degradation in more than one anatomical site for them to reach their target sites intact, and protecting a peptide or protein from degradation by one protease may not necessarily lead to marked increase in its stability or in the amount of peptide or protein reaching the site of action [13,14].

In the GI tract, protein digestion is initiated in gastric juice by a family of aspartic proteinases called pepsins. The mixture of peptides resulting from the partial digestion of proteins in the stomach will be acted upon by pancreatic proteases upon reaching the small intestine. Such proteases include trypsin, α-chymotrypsin, elastase, and carboxypeptidase A [15]. Further degradation will happen upon contact with the brush border and/or intracellularly depending on the resistance of a given peptide to the proteases in the brush border [9,15]

1.2.1.2. Physical barrier

The epithelial and vascular endothelial membranes play important roles in the delivery of drugs from the site of administration to the site of action, which for most drugs is located extravascularly. In the case of peptide and protein drugs, they are a major barrier to absorption. Among several epithelia, the intestinal epithelia are the best characterized in terms of peptide and protein absorption [16]. The mucosal surface of the digestive tract is a vast surface area covered by a monolayer of epithelial cells, joined by tight junctions that provide an effective barrier to proteins and peptides (Figure 1.1). The tight junction, which forms a band around the perimeter of the cell, separates the cell membrane into an apical surface (facing the intestinal lumen) and a basolateral surface [17,18]. This membrane is highly invaginated, forming microvilli which serve to increase the surface area available for absorption. The intestinal absorptive cell or "enterocyte" is not a uniform cell type; its phenotype changes during intestinal development of the fetus and neonate, and during differentiation of individual cells in adults.

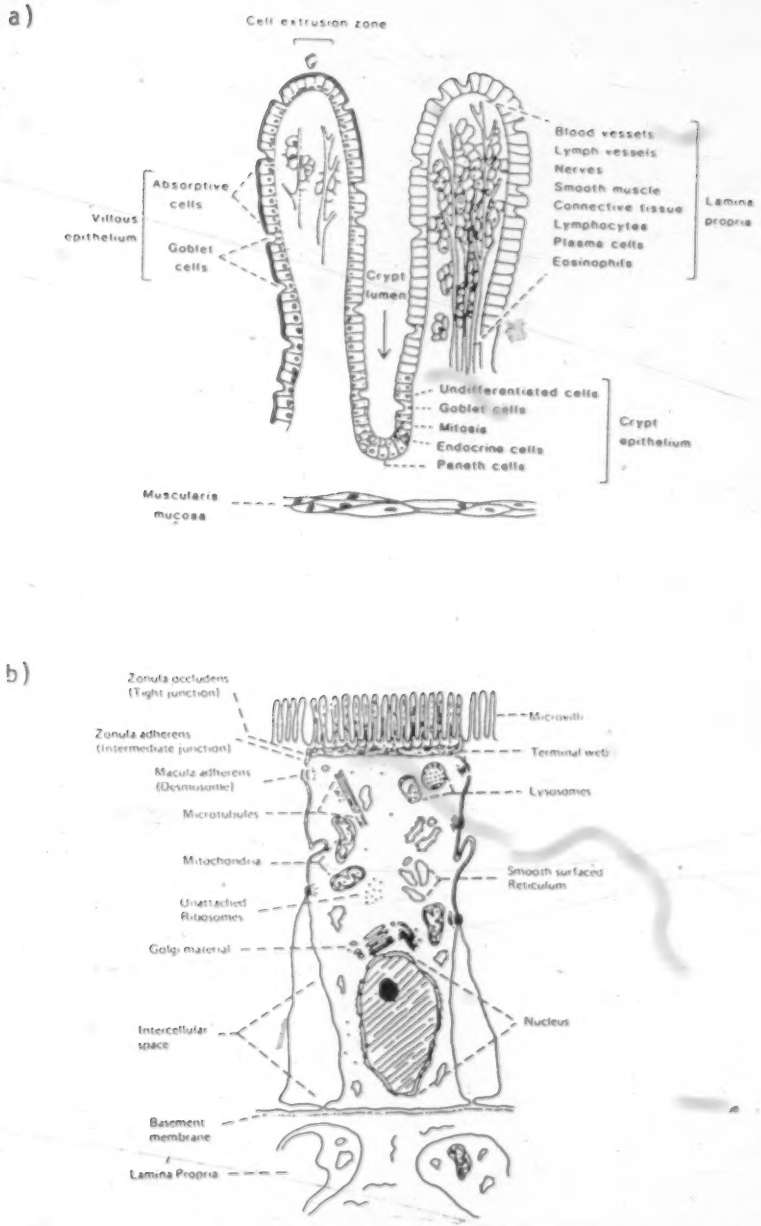


Figure 1.1. Schematic representations of (a) two villi and a crypt and (b) the organization of the organization of the intestinal epithelial absorptive cells (from reference 16).

Enterocyte phenotype vary widely in their capacity for endocytosis and transcytosis [19]. Over specific mucosal sites marked by the presence of subepithelial, organized lymphoid follicles (Peyer's patches), a unique epithelium occurs containing an unusual phenotype, the M cell. M cells are intermittently distributed among the absorptive cells covering the follicular dome. It has been proposed that they perform sampling and transport of undegraded luminal particles and macromolecules into lymphoid follicles for immunologic surveillance and initiation of appropriate immunologic responses [20]. Their functional significance for mucosal immunity is amplified by their unique position over the Peyer's patches, the inductive sites for mucosal immune responses [21].

Physical studies of cell membranes may help to better understand their functionality as barriers. The major structural motif of the cell membrane is the lipid bilayer. The lipids of cell membranes are quite heterogeneous of which the main components are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin, the simple glycolipids, glucosylceramide and galactosylceramide, and cholesterol. Studies of intestinal epithelial cells reveal that the microvillus membrane contains intramembrane proteins, fractions of proteins, or protein assemblies which exceed those found in many other plasma membranes [22]. High protein/lipid, cholesterol/phospholipid, and glycolipid/phospholipid ratios of 1.7:1, 1.22:1, and 1:1, respectively, in the microvillus membrane are in contrast with cholesterol/phospholipid and glycolipid/phospholipid ratios of less than 0.5:1 in basolateral membrane-enriched preparations of absorptive cells [23-26]. The high glycolipid and cholesterol content of microvillus membrane yields a low fluidity [27]

which allows them to withstand osmotic fluxes and the influence of bile salts in the harsh environment of the GI tract [28]. In addition, the surfaces of microvilli are covered by a mucus layer, which is a functional part of the unstirred water layer overlaying the intestinal epithelium and appears to represent a potential barrier to the mucosal transport of macromolecules [16].

1.2.1.2.1. Peptide and protein transport across the GI tract

Transport of peptides and proteins across the intestinal epithelium may happen through simple diffusion, active transport, endocytosis and transcytosis, and/or paracellular movement. Simple diffusion seems to be of the least importance in the absorption of macromolecules. Amino acids, di- and tripeptides are mainly absorbed by carrier-mediated transport systems. The peptides and proteins which are too large to be absorbed by active transportation may be taken up by endocytosis. The endocytotic uptake provides a means to overcome the mucosal barrier of the intestine epithelium. The process known as receptor-mediated endocytosis (RME) allows the absorptive cell of the intestinal epithelium to select and transport specific molecules while excluding undesirable or potentially harmful ones. It has been known for a long time that in mammals, mothers can transfer passive immunity to newborns through the intestinal absorption of immunoglobulins (Ig) from colostrum [29]. Other important examples are nerve growth factor (NGF) [30] and epidermal growth factor (EGF) [31], which have been shown to be absorbed following oral administration to suckling rats. Moreover, specific receptor-mediated binding and endocytosis of EGF was demonstrated in intestinal epithelial cells of suckling rat, mouse, and subcellular fractions of rat small

intestine enriched in microvillus membrane [32-35]. These results indicate that transepithelial transport may play a role in the mucosal transport of these macromolecules.

1.2.1.2.2. Paracellular transport

Cells of transporting epithelia have two basic characteristics: (1) they form tight junctions that confer to the layer of cells their ability to act as diffusion barriers; and (2) their plasma membrane polarizes into an apical and a basolateral domain, an asymmetry that enables them to transport substances vectorially. These cells are able to regulate the degree of attachment between themselves and control the amount and nature of the substances allowed to permeate the epithelium, either by traversing their cytoplasm or by crossing through the paracellular pathway. When two fluid compartments separated by the epithelium have similar compositions and the volume to be translocated is large, the cells, by controlling the permeability of tight junctions, allow an easy passage of substances through the paracellular pathway. Furthermore, the structure and permeability of the tight junctions and the paracellular pathway may be drastically changed in response to a variety of signals so as to adapt the epithelium to physiological requirements [36]. Within local regions of the small intestinal epithelium, morphological differences have been found that might indicate differences in epithelial permeability. It is important to note that the tight junctions between undifferentiated crypt cells are more loosely organized and are composed of fewer strands than those between the absorptive (villus) cells. This suggests that the crypt epithelium may be of a "leakier" nature than the villus epithelium [16]. There has also been a perception that this route is deficient in proteolytic

activity [7]. Several peptide drugs such as DDAVP (an analog of vasopressin) [37], thyrotropin-releasing hormone (TRH) [38] and octreotide [39,40] are assumed to permeate the intestinal mucosa mainly via this route. In summary paracellular spaces are potential routes for the transfer of peptide drugs from one biological compartment to another, and suitable materials that act as keys to open such routes should be found [36].

Since the paracellular pathway is an aqueous extracellular route across the epithelia, structural features that influence the paracellular absorption of peptide drugs are size, charge, and hydrophilicity. Of these structural features, molecular size probably plays the most significant role due to the size restriction of the junctional complexes [41]. Okumu et al. [42] have demonstrated, in vitro Caco-2 cell permeability studies with Asp-containing peptides, that cyclo-[Trp-Ala-Gly-Asp-Ala] is 2-3 times more permeable than Ac-Trp-Ala-Gly-Gly-Asp-Ala-NH_2. They concluded that cyclization may have decreased the average molecular size, accounting for the observed increase in flux. Another group of workers showed in a series of model peptides, DPheGly, DPhe2Gly, and Dphe3Gly that the flux decreased when molecular radius increased [43]. The effect of peptide charge on the extent of its paracellular movement is still controversial. Rubas et al.[44,45] have shown in a series of cyclic RGD peptides that those with a net charge of -1 or -2 exhibit optimum permeability for the paracellular transport. Another group of workers in a study with small anionic, neutral, and cationic non-peptide markers have found higher permeability coefficients for cationic species [43]. In contrast, studies with some model peptides revealed no discrimination in their flux on the basis of charge [40,46].

1.2.2. Approaches to Maximize the Oral Absorption of Protein Drugs

Improvement of oral bioavailability of peptide and protein drugs has essentially been based on strategies to overcome metabolic and/or physical barriers to the absorption of drugs in GI tract. Such improvements have been achieved to some extent by chemical modification of peptide drugs, co-administration of protease inhibitors, the use of penetration enhancers, or by control of drug release in novel formulation approaches.

1.2.2.1. Chemical modification of drugs

Chemical modifications tend to optimize not only potency and selectivity but, most importantly, stability of peptides toward proteolysis. These modifications include (1) substitution of L-amino acid with D-amino acid, (2) N -acetyl, -formyl, and other N -alkylations, (3) conversion of a terminal carboxy group to an amino alcohol, methyl ketone, nitrile, tetrazole or decarboxy amino acid, (4) conversion of linear peptides to cyclic analogs, (5) inverting the chirality of each amino acid, (6) attachment of fatty acid moieties to amino termini of peptides, hence improving their proteolytic stability and penetration, and (7) the use of prodrugs [7,9,41,47,48].

Depending on the site and mechanism by which a given peptide drug is metabolized, appropriate chemical modifications can be used to design metabolically-stable analogs. In addition, these modifications alter the overall lipophilicity of the molecule, which might also facilitate their penetration through biological membranes [41].

1.2.2.2. Co-administration with protease inhibitors

Co-administration of protease inhibitors has been shown to substantially promote the oral absorption of several peptides. The absorption of pentagastrin from the duodenum of the rat was enhanced 11.5 times when the pancreatic duct was ligated to exclude the pancreatic digestive enzymes from the small intestine [49]. The renin inhibitor, PHPFHLFVF was found to cross the adult rabbit jejunum 90% intact in the presence of phosphoramidon, a metallo-proteinase inhibitor [50]. In the absence of protease inhibitors, however, over 55% of this nanopeptide was degraded in 5 min and none was left after 30 min. Derivatives of α-aminoboronic acid, which are reversible inhibitors of aminopeptidases, markedly inhibited the hydrolysis of leucine enkephaline in the nasal cavity of the rat. Compared with bestatin and puromycin, boroleucin (one of the derivatives) was 100 and 1000 times more potent, respectively, and was effective at 0.1 μM [51]. Three oxytocin peptide analogues, CAT, F314 and F327, were absorbed with a bioavailability of about 0.5% in the presence of pancreatic juice, but increased to 1%, 2.1%, and 13.5% respectively, when the pancreatic juice was diverted from the intestine [52]. Bacitracin enhanced the nasal absorption of tetracosactide in the rat from 4 to 24%, compared with 12% afforded by a 1% Na glycocholate [53]. Coadministration of 2 mM puromycin, an aminopeptidase inhibitor, with metkephamid promoted its oral bioavailability in the rat from 0.22 to 4.5% [54]. Cytosolic insulin degradation in Caco-2 cells and rat intestinal enterocytes was strongly inhibited by insulin degrading enzyme (IDE) inhibitors [55,56]. Insulin absorption from the ileum of the rat was enhanced at a small dose of 3 mg soybean trypsin inhibitor [57]. The effectiveness of aprotinin and

soybean trypsin inhibitor in enhancing insulin absorption is augmented by co-administration with the bile salts deoxycholate and cholate, which when used alone were less effective than the protease inhibitors themselves in promoting oral insulin absorption [58]. This suggests that the enzymatic barrier is more of an impediment to insulin absorption than the membrane transport barrier [58].

In addition to the known protease inhibitors, sodium glycholate, a penetration enhancer, also inhibited leucine aminopeptidase activity and protected insulin from proteolysis in rat nasal homogenates [59]. Protection of insulin from proteolysis by sodium glycocholate is not limited to the nasal mucosa, but is extended to the buccal, rectal, vaginal, and ileal mucosae as well [60]. Moreover, the extent of protease inhibition is dependent on both the nature and concentration of the substrate [61,62]. Sodium glycocholate and other bile salts appeared to inhibit proteolytic activity by denaturing the enzyme and preventing the enzyme-substrate complex formed to undergo the necessary conformational change which aligns the catalytic site on the protease with the susceptible bond of the substrate [61].

The selection of peptidase inhibitors depends on the type of target protease and its subcellular distribution. Primarily membrane-bound protease activity can easily be suppressed by simple co-administration of an effective inhibitor. Where the principal proteolytic activity is cytosolic, however, it is imperative that peptidase inhibitors themselves be absorbed by the cell. This may be associated with significant problems, since such compounds are typically too large and too hydrophilic to partition into and diffuse across biological membranes.

Peptidase inhibitors have been recognized as offering great potential to reduce intestinal metabolism of peptides and, therefore, increase their oral bioavailability. However, a concern when using peptidase inhibitors to promote peptide absorption is their possible effect on the mucosal cell (e.g. change on the tight junctions) [63] and physiologic processes following absorption into the systemic circulation [64,65]. As in the case with penetration enhancers, it is imperative that the effect of the inhibitors on membrane integrity be tested [66].

1.2.2.3. Use of penetration enhancers

Penetration enhancers, substances which facilitate the absorption of solutes across biological membranes, have been widely used to improve the absorption of peptides and proteins in pharmacological amounts. Penetration enhancers include chelators, surfactants, bile salts, enamines, salicylates, acylcarnitines, acylcholines, acyl amino acids, fusidate derivatives, cyclodextrins, fatty acids and medium chain glycerides [2,28]. The mechanism by which enhancers act are poorly understood. Enhancers are believed to act by one of three mechanisms: (1) compromising integrity of the mucosal membrane, (2) inhibiting proteolytic activity, or (3) increasing the thermodynamic activity of peptide and protein drugs [2]. These low molecular weight compounds enhance drug penetration by lowering diffusional resistance of either or both of the transcellular and paracellular pathways [9,67].

Enhancers may increase transcellular permeability to peptide and protein drugs by perturbing integrity of the membrane and removal of lipids [68,69], proteins [70] or

nonprotein thiols [71]. Thus, some investigators have attempted to correlate absorption-promoting potencies of enhancers to the extent of damage to the membrane, usually assessed by measuring release of lipids and proteins from a given mucosa. One should bear in mind, however, that such correlations do not always hold true. For instance, it was demonsterated that the three-fold increase in phospholipid release from the isolated colonic loop was not an important factor in the enhancement of salicylate absorption by caprate [72]. Enhancement in paracellular permeability has been postulated as being due to expansion of pore size of the mucosal membrane [69], and increase in water influx through that space [73,74]. Lowering extracellular Ca^{2+} concentration, enhancing Na^{+} transport by increasing osmolality of the dosing solution or by promoting glucose and amino acid transport, and altering actin filaments by compounds such as cytochalasins and phorbol esters promote permeability of tight junctions. Alternatively penetration enhancers may work by induction of endocytosis, indicated in gene transfer as well as facilitation of macromolecular transport across the blood-brain barrier and the intestinal epithelium [75-77].

NSAIDs, monoglycerides and sodium caprylate dimethyl maleate mainly enhance penetration of drugs through the transcellular pathway. Chelating agents such as EDTA, Na taurocholate, Na laurate, and mixed micelles of Na elate and Na taurocholate do so through the paracellular pathway. Na glycholate, Na caprate, Na deoxycholate, and polyoxyethylated nonionic surfactants seem to affect both pathways [67]. The efficacy of penetration enhancers is influenced by several factors: i) physicochemical property of the enhancer and its mode of action relative to the peptide or protein drug, ii) the

pharmacokinetic profiles of the drug and enhancer (i.e. reaching therapeutic concentrations maintained for appropriate periods of time in their therapeutic compartments, e.g. drug in blood and enhancer in intestine, iii) site-to-site variation in effectiveness of enhancers, iv) physicochemical properties of the vehicles, and whether they are used alone or in combination. Also there are some safety concerns associated with the use of penetration enhancers which need to be considered and addressed appropriately. These are irritation of tissues, nonspecifity in action, causing membrane damage, adverse effects on ciliary movement, and absorption of the enhancer into the blood circulation [2,28].

1.2.2.4. Formulation and drug delivery approaches

The other strategy to improve oral absorption of peptide and protein drugs is to protect the drug in the harsh enzymatic environment of GI tract, to prolong drug residence time in the GI tract or specifically at the site of absorption, and/or to target the drug to an optimal region of the GI tract where its absorption is maximal. Different regions of the GI tract, from the small intestine to the colon, vary in membrane thickness, surface area, cellular structure, pH and enzyme content. This has prompted scientists to design and optimize formulations for the oral delivery of a variety of peptides to selected regions of GI tract.

1.2.2.4.1. Small intestine

The absorption capacity of the GI tract decreases serially along the duodenum, jejunum, ileum, and large intestine. Although there are species differences in the regional

distribution of aminopeptidase activity, the proteolytic activity of ileum is higher than jejunum regardless of species differences [78,79]. This can be partly responsible for the higher absorption of peptides such as Met-Met or Gly-Gly from the jejunum when compared with the ileum [80]. However, the ileum is leakier than other parts of the intestine and allows a more favorable absorption profile for some peptides. The results obtained by Michel et al. [81] and Morishita et al. [82,83] suggest that the ileum has a higher potential for absorption of insulin than other parts of the intestine. The ileum also contains highly specialized mechanisms for absorption of specific macromolecules such as cyanocobalamines.

1.2.2.4.2. Colon

The colon has been investigated as an alternative region for targeting orally-administrated peptide drugs. There have been several concerns with the colon as a primary drug delivery site, such as an extremely variable pH and a small surface/volume ratio when compared with other segments of the GI tract [84]. Other concerns are that in the colon, drug is surrounded by fecal matter which restricts free diffusion to the mucosal surface, and that the concentration of bacteria, largely anaerobic species, is several orders of magnitude higher than in the small bowel. This rich bacterial enzyme population may potentially lead to faster drug degradation. However, slower mucus turnover rates (thus stronger mucoadhesive forces between polymer and mucus, in the case of using mucoadhesive drug delivery systems) [85], lower enzyme levels, especially peptidases, than in the small intestine [86] and longer residence times of 10-20 h [87] have prompted researchers to view the colon as a potential site for peptide drug delivery. Many

pharmaceutical excipients and drug delivery systems such as azo cross-linked polymers, polyurethanes of diisocyanates and sugars, galactomannans, dextran esters, and amylose have been employed to achieve this goal [88-90]. Interestingly, microbial enzymes have been exploited to promote the colon as a drug delivery site. Saffran et al. coated a protein with a copolymer of styrene and hydroxymethylmethacrylate cross-linked by an azo bond. This system remained intact throughout the GI tract until arrival at the ileocecal junction, whereby, azoreductase secreted by the indigenous bacteria reduced the azo bond, causing the polymer to disintegrate and release encapsulated protein for absorption [63,91]. This concept has attracted many investigators to the area of oral delivery of peptide drugs [84,92,93]. However, further systematic approaches towards the design of such systems are required if azo polymers are to offer a successful, specific colon drug delivery system[94]. Another group of researchers have tried to solve the low permeability problem of the colon by using penetration enhancers, and reported that the rat colon was more sensitive than the small intestine to the effect of penetration enhancers [82].

1.2.2.4.3. Peyer's patches

Peyer's patches, rectangularly or ovally-shaped lymphoid follicles, situated on the mesenteric border of the small intestine encompassing the M-cells, are believed to be a port of entry for lymphokines and, thus, have been the focus of researchers for studying oral immune induction. Ovalbumin (OVA) entrapped in an antigen delivery system comprising poly(D,L-lactide-co-glycolide) (PLGA) microparticles, showed the mean salivary IgA titre at week 6 of 959 ± 494 U compared with 30 ± 5 in the soluble.OVA group ($P<0.0001$). The authours concluded that microparticles were taken up by antigen-

presenting cells in Peyer's patches, then slowly degraded in vivo and released entrapped antigens, and thus were able to function as potent antigen delivery systems. This gave both mucosal and systemic responses [95]. Oral delivery of vaccines using alginate microspheres elicited the production of secretory IgA (sIgA) at the mucosal surfaces in mice, rabbits, and cattle. Oral vaccination of chicken resulted in an increased delayed-type hypersensitivity, a cell-mediated immune response, indicating a positive response to the vaccine [96]. It was suggested that even though alginate microspheres adhered to Peyer's patches, selective absorption alone could not explain the results of their study. Since the microparticles used had a size range of 1 to > 30 μm, the release of antigen and its selective absorption in the intestine could not be differentiated from that of microparticles [96]. In vitro incubation of rat Peyer's patches with liposomes which were either uncoated or coated by the reovirus M cell attachment protein sigma 1, resulted in a 10-20 fold higher uptake of the coated liposomes. These results suggest that selective adherence of a carrier system to M cells may facilitate delivery of carrier contents to mucosal underlying lymphoid tissue, thereby enhancing the immune response [97]. In a study by Childers et al., surgically constricted segments of rat small intestine were exposed to small unilamellar liposomes or gold-labeled solid core liposomes. Transmission electron microscopy of Peyer's patch showed M cells with endocytic vesicles containing liposomes. Such vesicles containing liposomes were also observed between M cells and lymphoid cells. These results indicate that intact liposomes can be endocytosed by M cells and provide evidence for a possible mechanism by which M cells deliver antigen to lymphoid cells in Peyer's patches, supporting the potential usefulness of

liposomes in oral vaccine development [98]. Following the oral administration of liposomes to rats, 6-carboxyfluorescein and rhodamine B-PE encapsulated DSPC-liposomes were preferentially taken up by Peyer's patches in the lower ileum. The uptake of liposomes larger than 374 nm mean diameter was high. Orally-administered DSPC liposomes of a large diameter thus appeared to serve effectively as a vehicle for delivering antigens to Peyer's patches [99,100].

1.2.2.4.4. Lymphatic system

The lymphatic vessles of villi join a network of capillaries in the grandular layer of the mucosa and are linked to collecting lymphatics which constitutes the beginning of the lymphatics of the mesentery. Since the junctions are widely separated and lymphatic capillaries have a fragmented basement membrane the lymphatic system exhibits little barrier towards the passage of solutes, fluids, and large particles [15,101,102]. There seems to be a linear relationship between the extent of lymphatic absorption and the molecular weight of the absorbed species [103]. Molecules with M.W > 16,000 are predominantly absorbed by the lymphatics that drain the application site [104]. There is some evidence that microparticles may target drugs to the lymph nodes. Following serosal injection of pepleomycin-coated activated carbon 167 nm particles into the anterior wall of the greater and lesser curvatures of the stomach antrum, 6-33 times higher pepleomycin concentrations were detected in the lymph nodes located along the left gastric artery and the right gastropiploic artery. By comparison, plasma levels were very low [105]. Lipid vehicles have also been considered to promote the lymphatic absorption of orally-administrated drugs. Two hours following oral administration of cylosporine A

(CsA) incorporated in 191 nm lipid microspheres composed of olive oil, soybean oil, egg lecithin, and glycerol, the CsA thoracic lymph concentration was approximately 46 times greater than from conventional preparations [106]. The enhancement of lymphatic absorption would be advantageous for those drugs whose target is in the lymphatic system.

1.2.3. The Liposome as a Drug Delivery System

Over the years, several drug delivery systems have been explored to protect insulin from proteolysis, localize an insulin formulation in a specified region, and/or prolong the drug residence time in the GI tract. They include water-in-oil and water-in-oil-in-water emulsions [107], nanoparticles [108], gel spheres [109], microtablets [110], capsules coated with polyacrylic polymers [111,112], and liposomes [113-116].

The liposome has been widely employed because of its ease of preparation, versatility of its composition, possibility of using mild conditions of preparation for peptides, and its biocompatibility. The concern with oral administration of liposomes has been their relatively poor stability in the GI environment. A great challenge in liposome drug delivery has been to develop effective liposome formulations tailored for oral administration. Coating liposomes with polymers has been shown to improve their GI stability and control drug release [117,118]. More recent efforts involving Ara-C, a small molecule drug, have shown that reduced drug leakage rate and greater liposome stability in GI tract may be achieved if liposomes are coated by cholesterol derivatized polysaccharides rather than polymers having no hydrophobic anchor [119]. The

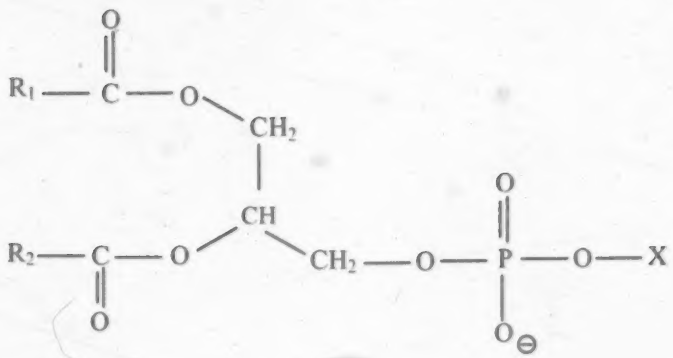
cholesterol moiety on the polymer can penetrate into the bilayer resulting in a stronger polymer-lipid interaction and producing more stable liposomes, while the hydrophilic part of the polysaccharide may provide a closer contact of the formulation with the absorbing surface of the GI tract, resulting in the protection of drug from luminal enzymes and improving its absorption. The following section provides a background on liposomes and their characteristics pertaining to a drug delivery system specifically for peptide drugs and also describes attempts to prepare functionalized liposomes for the oral delivery of insulin.

1.2.3.1. Properties/characteristics of liposomes

Liposomes are microparticulate lipoidal systems consisting of one or more bilayers arranged concentrically with aqueous compartments and core. They are made predominantly from naturally-occurring phospholipids and are, therefore, biocompatible, i.e. biodegradable, nontoxic, and do not cause immune response. Introduced by Bangham and Horne in 1964 [120], liposomes have been considered to be excellent models of cell membranes as well as useful carriers of both lipid-soluble and water-soluble drugs. Advantages of liposomes extend to reducing toxicities of encapsulated drug [121-124], targeting to specific tissue sites [125-127], enhancement of drug efficacy or potency [123,128], and sustained release of drugs [129].

1.2.3.1.1. Composition

Phosphatides comprise the major bulk material commonly used for liposome construction, shown below.



The groups designated R_1 and R_2 represent hydrocarbon chains that typically range between 11 and 19 carbon units, with the 16, 18, or 20 carbon fatty acyl groups possessing two or three double bonds being most commonly found in nature. The designation "X" refers to head group substitution including choline, hydrogen, ethanolamine, serine, inositol, and glycerol. Differences in the acyl chains and the head group result in phosphatides having different physical properties, notably their gel and liquid-crystalline phase transition temperatures (T_m) and various amphipathic and ionic characteristics.

Phosphatidylcholines (PC), also called lecithins, are the most widely used class of lipids. They are zwitterionic at physiological pH values and have limited solubilities in water but spontaneously form colloidal dispersions. In aqueous media they align themselves tail-to-tail in planar bilayer sheets in order to minimize unfavorable exposure of hydrocarbon chains to the aqueous environment. Upon further hydration, bilayers grow and form tubular fibrils, greatly increasing the contact area with water. Upon agitation

these tubular structures detach and immediately seal off their exposed edges and form multilamellar vesicles (MLVs) [130].

A variety of non-phospholipid lipids have been added into formulations with the intent to alter either surface charge, fluidity, size or lamellarity of the vesicles. A common additive is cholesterol (CH) which is often used to stabilize liposomal membranes. Additionally, ingredients such as dicetyl phosphate (DCP) and stearylamine (SA) are often used to impart negative or positive charge, respectively, to the membrane. Charged liposomes may also be prepared by employing various phosphatides. For example phosphatidylserines (PS), phosphatidylglycerols (PG), or phosphatidic acid (PA) can be employed to render liposomes having a negative surface charge.

1.2.3.1.2. Phase behavior

At different temperatures, phosphatides can exist in different states: gel or liquid crystalline state [131,132]. Phase transitions bring about drastic changes in the properties of liquid crystals and they can be induced by a number of factors, such as temperature, electric fields, ions, solutes, and pH [133]. The temperature of this transition, T_m, at which frozen and highly ordered hydrocarbon chains melt into a fluid, is an extremely important physical characteristic of liposomes. Indeed, the phase transition and fluidity of phospholipid membranes determine such properties as packing, permeability, fusion, aggregation, protein binding, all of which can markedly affect the stability of liposomes, and their behavior in biological systems [134]. As liposomes are heated, the packing defects will increase both in duration and in number giving rise to a temperature-

dependent increase in membrane permeability to small ions and polar molecules. At the T_m both the gel and liquid-crystalline phases co-exist in different parts of the same membrane, and a consequent large increase in packing defects is found at the interface of the two phases [135]. These defects are mainly boundaries between solid and liquid phase and bilayer dislocations [134]. As a result, membrane permeability at phase transition is the highest, compared to either higher or lower temperatures than the T_m [135].

Addition of CH is known to increase disorder in the low-temperature phase and reduces the intensity of the phase transition. At higher CH concentrations (30-50 mol%) the transition is practically eliminated [136]. CH decreases membrane fluidity > T_m by contributing to stronger Van der Waals forces of attraction [137] and the opposite holds < T_m of the phospholipid.

1.2.3.1.3. Bilayer permeability

Permeability of liposomes is strongly dependent on the dynamics and phase behavior of the bilayer. Permeation is accompanied with structural changes including structural disorder in the membrane. For solute molecules with high solubility in both organic and aqueous solvents, a phospholipid membrane clearly constitutes a very tenuous barrier. On the other hand, polar molecules, such as glucose and higher molecular weight compounds, pass across the membrane very slowly. Since phospholipid membranes are semi-permeable, a concentration difference of solute on either side of the membrane generates an osmotic pressure which leads to a net transfer of water molecules on one side of the membrane. This may result in an accelerated leakage or even total loss of the

content of liposome into the bulk aqueous phase due to complete rupture of the membrane. The permeability of the membrane to entrapped hydrophilic low molecular weight solutes depends on size and charge of the solute entrapped, physical state of the membrane, environmental factors, such as temperature, pH, ionic strength and chemical content of the medium and, in the case of in vivo the effects due to enzymes and bile salts [134,135].

Permeability has been proposed, in some cases, to follow bi-phasic first order kinetics [138-140]. First-order kinetics may be a good assumption in most of the studies, however, several exceptions exist. Zero order kinetics may be followed when the membrane is not release rate determining. In instances that permeation involves a special mechanism such as a bimolecular event, second-order kinetics may be followed. In practice, however, kinetic reactions may follow any non-integer order, although first-order kinetics is more often the closest approximation [134].

1.2.3.1.4. Size

Many physicochemical and biological properties of liposomes, such as the amount of entrapped volume per given amount of lipid, leakage rates, adhesion, aggregation, stability during absorption, biological-fate, and the extent of clearance from the blood depend on liposome size. Liposomes of different sizes often require different methods of manufacture, and different applications often demand the use of liposomes within particular size ranges. Classification of liposomes according to size is the most common

index of characterization in current use. They range in size from about 20 nm to about 5 μm and above. Liposomes are described in one of several categories:

- Small unilamellar vesicles (SUVs). These are at the lowest size limit (20-100 nm) which varies slightly according to the ionic strength of the aqueous medium and lipid composition. They exist in a relatively homogenous population in terms of size.

- Large unilamellar vesicles (LUV). These liposomes range in size from about 100-500 nm.

- Multilamellar vesicles (MLVs). These usually consist of a population of vesicles covering a wide range of sizes (100 nm-10 μm), each vesicle generally consisting of five or more concentric lamellae.

In practice, most methods of liposome preparation give a fairly heterogeneous population of vesicles with a wide distribution of sizes, particularly towards the lower end of the range. It is important to characterize liposome preparation according to size in order to properly interpret their *in vitro* or *in vivo* behaviors [141].

1.2.3.2. In vivo behavior of liposomes

The fate of parenterally-administered liposomes may be influenced by composition, size, charge, and surface hydrophilicity of vesicles, by plasma constituents such as lipoproteins and phospholipases, and by the extent and types of interactions with cells [134,142]. Two separate mechanisms for neutral liposomal uptake have been proposed: i) interactions with plasma components such as lipid exchange and depletion leading to

liposome disintegration and, ii) opsonization followed by macrophage uptake [142]. Improved circulation time of liposomes and/or the entrapped drug can, therefore, be achieved by reduction in the rates of these reactions [142]. A preliminary estimation of in vivo release of drug may be obtained from incubation in plasma, the so-called plasma-induced leakage assays [143,144]. In fact, for any application drug release may be assessed by measuring the leakage of an indicator or drug from liposomes in the desired media such as a simple buffer, enzyme, or detergent solution [145].

Successful *in vivo* drug delivery via liposomes requires maintaining physical stability long enough to reach target sites or serve as a depot of drugs. Many workers have tried to improve liposome stability or targetability for *in vitro* or *in vivo* applications. The attempts generally have included preparation of polymerized or surface-modified liposomes.

1.2.3.2.1. Polymerized liposomes

Polymerized liposomes are prepared normally by UV irradiation of aqueous dispersions of lipids having polymerizable groups either in the hydrocarbon chain or in the polar head group region [146,147]. The thermotropic properties of the membranes are usually changed appreciably after polymerization. Most of the bilayers lose the fluid phase in the absence of main and side chain spacers due to polymerization. As a result, the stability of liposomes is greatly increased. Because the issue of toxicity of these compounds in vivo is largely unknown, many researchers work with natural or

unpolymerized synthetic phospholipids and employ other modifications to improve liposome stability.

1.2.3.2.2. Surface-modified liposomes

Liposome surfaces have been modified to improve in vitro and in vivo stability or targetability thereby altering biological fate and pharmacokinetics of liposomes. Conventional liposomes, which are mainly composed of phospholipids without any surface modification, are quickly taken up by the MPS after i.v. administration. Thus, they may be considered as excellent drug carriers for diseases associated with MPS and in applications as immunomodulation or vaccination. Thus, there have been a great deal of efforts to finding ways to reduce liposome MPS uptake and effectively prolong their blood circulation time and/or to target them in vivo to cells other than MPS cells [127,142,148-150].

Targeting may be classified into two categories of passive or active targeting. In passive targeting with a bulk recognition mechanism, the targeting (recognition of a specific tissue cell) is attained by modification of the liposome surface charge, size, membrane fluidity and hydrophilicity. With active targeting, recognition is at the molecular level through direct and specific interaction between a specific recognition site on the liposomal surface and a receptor on the cytoplasm membrane of the target cell [125,151,152]. Incorporation of antigens, antibodies, and specific molecular ligands makes liposomes targetable. For example, lectins composed of glycoproteins which specifically bind to carbohydrates are frequently used ligands. There has been evidence of

galactose-specific lectins on the membrane of several human tumor cells, such as Hela, hepatoma, and melanoma [125]. Tomato lectin, an apparently non-toxic dietary glycoprotein, was shown to bind avidly to rings of small intestine and this binding could be inhibited with specific competing sugars. The specific binding of tomato lectin to the enterocyte cell surface has the potential for increasing gastrointestinal residence times of an oral drug formulation [153].

Principles of stabilization of lipid bilayers by polymers are outlined schematically in Figure1.1. The four different approaches have been identified as [146]:

1. adsorption of polymers to the liposomal surfaces through hydrophilic and/or ionic interactions;
2. attachment of polymers having hydrophobic substitutions which penetrate the lipid bilayer;
3. fixation of polymerizable water-soluble monomers to the vesicle surface via salt formation, followed by an initiated or spontaneous polymerization;
4. adsorption of polymerizable, water-soluble molecules on liposome surfaces and cleavage of the spacer groups after polymerization.

Synthetic Coated Vesicles: Liposomes in a "Basket" (Schematic) Coating of Liposomes by a Non-covalantly Linked Polymeric Network

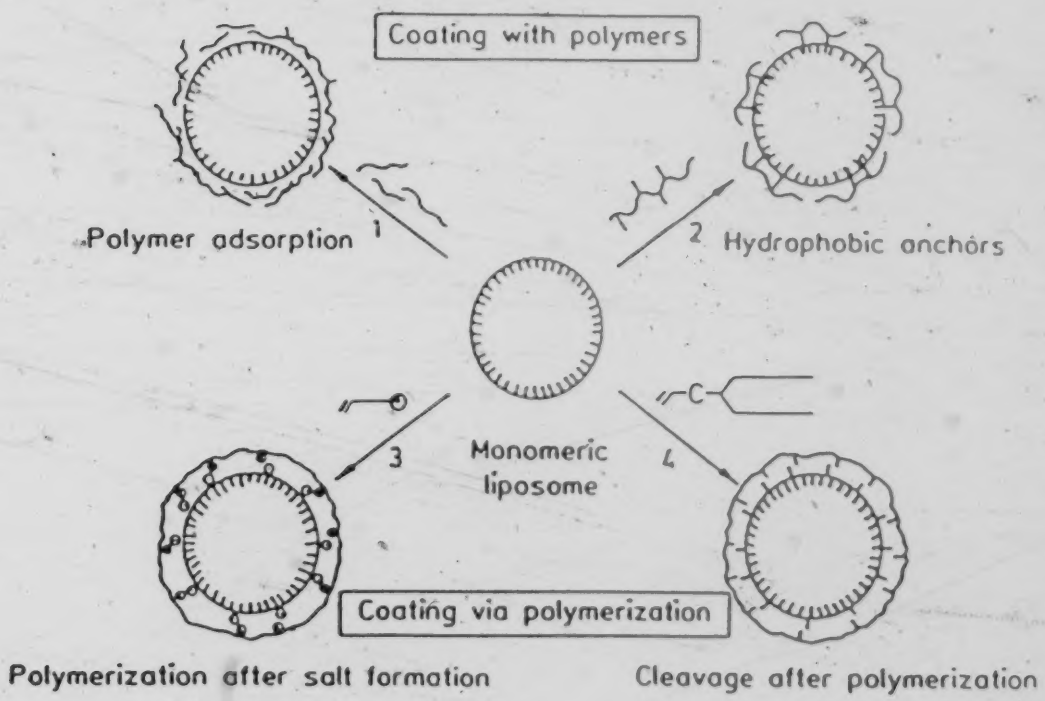


Figure 1.2. Possible approaches to stabilize liposomes via surface coating with polymers (from reference 146).

Coating liposomes with polymers via hydrophilic adsorption is performed by incubation of polymer solution with pre-formed liposomes. To enhance the interaction of polymer and liposomes, the reverse-phase evaporation technique has been employed to deposit the polymer at the surfaces of liposomes during their formation. Carboxymethyl chitin has been successfully used to coat liposomes by this method [154-157]. Another method used is microencapsulation of liposomes via a coacervation technique where the liposomes are mixed with the polymer (e.g. acacia-gelatin) solution at 40°C followed by emulsification in an organic solvent to form a water-in-oil emulsion. The temperature of the emulsion is adjusted to 10°C and the formed microcapsules are hardened with formaldehyde [118,158,159]. The preparation of liposomes microencapsulated by a wall of poly-L(lysine) for pulsed-release of entrapped BSA has been reported [160]. The advantage of this method is the ability to coat liposomes with polymers which do not have a specific functional group, however, precautions must be taken to avoid possible deterioration of liposomes due to contact with organic solvents.

Coating liposomes via salt formation is performed by autopolymerization of monomers carrying an opposite charge of the liposomes. Salt formation between cationic 4-vinylpyridine (4-VP) and negatively-charged liposomes followed by autopolymerization of 4-VP under normal conditions produced poly(4-VP)-coated liposomes. These liposomes had greater stability in isopropanol and sodium cholate solutions and showed reduced leakage rate of the encapsulated drug [117]. This procedure is relatively easy and the kinetics of the polymerization process can be followed by UV spectral analysis. A possible disadvantage of this approach is the inconvenience of removing the unreacted

monomers before the preparation could be used clinically and their possible toxicities [161,162].

Coating liposomes by polymers bearing a hydrophobic anchor group has been proposed to achieve both stability and active targetability of liposomes in vitro and in vivo [125]. Poly(carboxylic) acids [163] or naturally-occurring polysaccharides such as dextran, pullulan and mannan [125,164-166] have been derivatized with hydrophobic anchors such as CH which is firmly held within the liposomal bilayer. Such an anchor stabilizes the polymer on the outermost surface of the bilayer and prevents its desorption upon dilution of liposomes. This assists in the targetability of liposomes, controlling drug leakage from the vesicles, and improving stability. It was demonstrated that hydrophobized dextrans protected liposomes against degradation by detergents, decreased the fluidity of the membranes, prevented fusion of the liposomes and enhanced stability [167]. An artificial bilayer membrane consisting of hydrophobically-modified polymers anchored onto small unilamellar vesicles has been shown to respond reversibly to changes in temperature. The polymers not only strengthen the bilayer, but also trigger controlled diffusion motions within the lipid chains [166].

1.2.3.3. Liposomal formulation of protein/peptide drugs

Broadly speaking, the therapeutic use of any drug including peptides or proteins, is a function of its duration of action and its physicochemical properties which altogether dictates selection of a suitable delivery system and the most appropriate preparation technique [2]. As a general precept, peptides and proteins are difficult materials to

pharmaceutically formulate and processing may cause protein/peptide deactivation [168]. Hence, care is needed in the choice of formulation ingredients and preparative conditions.

1.2.3.3.1. Peptide/protein drug properties considerations

Processing and formulation conditions such as temperature, pH, ionic strength, and composition may give rise to physical or chemical alterations [169-171]. Another important characteristic of peptides and proteins is their interaction with surfaces which may result in considerable loss and/or conformational changes. The degree and reversibility of the latter are dependent on time, temperature, agitation, and type of surface [169,170]. Broadly speaking, few stability problems should be expected if peptide or protein samples are kept cool, near neutral pH, and with minimal exposure to air [169]. Biological activity is obviously the most important property of a pharmaceutical peptide or protein and can be the indication of protein stability. However, an important point in the case of protein drugs, is that even though functional or full biological activity is strongly-dependent on its native three-dimensional structure, major changes in physical or chemical properties can be well-tolerated in some cases, producing only minor losses in assay activity, whereas in other cases very subtle physical alterations result in significant losses of biological activity. Therefore, the consequences of alterations of structure or conformation of a particular protein must be investigated on a case-by-case basis to determine if it pertains the stability, performance and safety of the drug [170].

1.2.3.3.2. Liposome preparation techniques

Nowadays, a wide range of different methods for the preparation of liposomes are available leading to a variety of vesicle types and sizes. Each vesicle type has advantages and shortcomings that should be considered with respect to the physico-chemical properties of the drug and the individual application. From the peptide drug point of view, those processes which avoid using organic solvents are preferred. This eliminates a number of difficult variables in the manipulation of proteins, including precipitation effects and deactivation [168].

The Classic-film MLV Method, first described by Bangham et. al [172], is perhaps among the most gentle on peptide drugs and is simple to conduct. Technically, the process involves the formation of a thin lipid film on a round-bottom flask by rotoevaporation of a solution of phospholipid, usually in chloroform, followed by hydration of the film by an aqueous solution of drug. The resultant liposomes may be stored at room temperature for a long time. Lipophilic drugs are incorporated into the dry film by co-dissolution in the organic phase. Being a gentle procedure adaptable to most drugs, including peptides and proteins, liposomes prepared by this method have a relatively low encapsulation efficiency. For this reason modifications and alternatives have been developed as discussed below.

The Freeze-and -Thaw (FAT) method is based on repetitive freezing-thawing of MLVs which causes breakage and reformation of the membranes and enhances loading of the drug from aqueous solution. Efficiency of drug encapsulation in this manner ranges between 30-50 percent but up to 90 percent is possible when high lipid concentrations are

used [173,174]. A disadvantage of this method is that the external medium must contain no amount of cryoprotectant agent. Active fusion of the membranes during FAT cycles is inhibited by a variety of agents like disaccharides [175,176] and certain amino acids [177]. The latter could be troubling when using this technique for a protein and peptide drug formulation. Another disadvantage is that many peptides and proteins can aggregate or fragment during FAT cycling [178]. In comparison *the freeze-dried technique* removes water via lyophilization which has been shown to enhance peptide encapsulation efficiency (EE) in liposomes. Drug loading can be accomplished either by drying a mixture of drug and lipid solution (in an organic solvent, e.g. chloroform) or by rehydrating freeze-dried vesicles in a solution of the drug [179]. It is important to evaluate the effect of lyophilization on physico-chemical stability and bioactivity of the peptide [178].

Osmotically-derived Vesicles is another technique which has been developed for preparation of peptide liposomes without using solvent. This method, like FAT, is based on deformation /reformation mechanism, derived by an internal osmotic pressure rather than lipid-phase transitions. A dispersion of liposome is made in the presence of a high concentration of osmotically-active agent such as NaCl or glucose. Preformed liposomes are then mixed with a solution of the same osmotic agent containing the drug at an equal weight/weight ratio with the lipid. The mixture is pumped in a circulating loop containing a tangential flow filter that allows filtration of the osmotic agent while retaining the liposomes and protein in the circulating loop. As the osmotic agent is filtered, a hypotonic washing solution is gradually introduced into the loop. During the gradual exposure to

hypotonic wash solution, liposomes will repetitively swell, rupture, and reform depending on the rate of removal of filtrate and rate of introduction of the hypotonic solution. A disadvantage of this method is that high concentrations of osmotic agents especially salts may be harmful to proteins [178].

Solvent-requiring Methods have also been employed to prepare peptide liposomes. Many preparation techniques of liposomes such as injection methods (ethanol injection, ether injection), demulsification methods (reverse phase evaporation and double emulsion), stable plurilamellar vesicles (SPLV) and the monophasic vesicle process (MPV) require the use of solvent. The solvent-requiring techniques mentioned above when applied to peptide and protein drugs result in high EE liposomes. However, damage to protein drugs during the manufacturing process is a likely possibility. Initial solvent screening to avoid loss of protein bioactivity and further consideration of the type of processing is essential.

1.2.4 Insulin

1.2.4.1. Pharmacology

Insulin is a hormone secreted by the beta cells of the pancreatic islets of Langerhans. It stimulates carbohydrate metabolism in skeletal and cardiac muscle and adipose tissue by facilitating transport of glucose into these cells. The hormone also stimulates protein synthesis and lipogenesis and inhibits lipolysis and release of fatty acids from adipose cells [180].

Insulin is used as replacement therapy in the management of diabetes mellitus. It supplements deficient levels of endogenous insulin and temporarily restores the ability of the body to properly utilize carbohydrate, fats, and proteins. Insulin therapy is indicated in all cases of insulin-dependent (type I) diabetes mellitus and is mandatory in the treatment of diabetic ketoacidosis. Insulin is also indicated in patients with noninsulin-dependent (type II) diabetes mellitus when weight reduction and/or proper dietary regulation have failed to maintain satisfactory concentrations of blood glucose in both the fasting and postprandial state [180].

1.2.4.2. Physicochemical properties

Insulin has a molecular weight of about 6000 and is composed of 51 amino acids in two chains connected by disulfide linkages. The amino acid sequence of the insulin molecule varies with the animal sources. Having a pI of 5.6, insulin is soluble at both acidic and physiologic pHs.

Insulin, like other peptide and protein drugs, possesses a large number of functional groups and, therefore, may undergo a variety of chemical or physical alterations with time, depending on storage conditions. The changes that occur may or may not affect the safety and efficacy of the protein as a drug [170]. It may be degraded non-enzymatically by various chemical reactions, de-amidation being the most prevalent [181]. In this reaction, the side chain amide groups of glutamine and aspargine are hydrolyzed to form free carboxylic acids [182,183]. The rate of de-amidation is dependent upon temperature, pH, ion strength, primary, secondary, and tertiary structure, and special intermolecular

interaction [182]. Physical alterations such as conformational changes and aggregation may also affect stability and bioactivity of the protein. Insulin may exist as monomers, dimers, hexamers, and higher oligomers, all at equilibrium in a neutral pH solution [184]. Aggregation leads to reduction in biological activity and obstruction of delivery routes [185]. In contrast, larger aggregates (hexamers) of insulin have lower degradation rates than smaller aggregates (dimers) when exposed to proteolytic enzymes [186]. This arises from the fact that smaller species offer a correspondingly larger number of exposed peptide linkages susceptible to enzyme attack [186].

1.2.4.3. Assay

Assay of insulin has been carried out in many ways utilizing visible spectrophotometric absorbance measurement (e.g. Lowry method), radiochemical method (e.g. γ counting), biological activity measurements (e.g. decrease of blood sugar or radioreceptor assays) and chromatographic methods. Today, HPLC is probably the most versatile and widely applied method for separation, identification, and quantification of peptides and proteins [187]. Reverse-phase HPLC has been widely used to separate and quantify insulin and its degradation products [114,181,186,188].

1.2.4.4. Route of administration

Parenteral administration of insulin is associated with physical and social discomfort. This may discourage many diabetics from accepting intensive insulin therapy, and some Type II diabetics to completely withdraw from insulin treatment [6]. In addition,

parenteral administration brings about a non-physiologic exposure of the body to the hormone [189,190]. Under physiologic conditions, insulin is secreted from ß-cells of Langerhans into the portal vein and the liver is exposed to much higher concentrations of insulin than peripheral tissues, whereas s.c. injected insulin results in higher exposure of peripheral tissues to insulin than liver; a nonphysiological condition [189,191-195]. Insulin was found to restore hepatic glucose production and levels of alanine and lactate with a much smaller dose in portal delivery than from peripheral administration [191-193]. Portal delivery was also shown to decrease the insulin dose required in long term glycemic control compared to peripheral routes [196]. In addition, insulin levels from administration through portal vein were 50 percent lower and return to fasting glycemia was faster than from administered peripherally [197]. On the other hand, oral administration of insulin provides physiologic transport of insulin because of its introduction to the liver prior to distribution to peripheral tissues and it will, inherently, be more accepted by patients.

1.2.5. Oral Administration of Liposomes

There have been many attempts to use liposomes orally to deliver drugs, including peptide and proteins. The harsh environment of the GI tract along with absorption-limiting factors are the main criteria limiting absorption and stability of drugs and/or delivery systems. Biological barriers to oral absorption of peptide and protein drugs and possible approaches to overcome them have already been discussed (sections 1.2.1 and 1.2.2).

The stability of liposomes as an oral drug delivery system is affected by a variety of factors, including the low pH of the stomach, and the presence of bile salts and enzymes, such as phospholipases, after gastric emptying. One approach to improve liposome stability has been to use phospholipids with high T_m and/or to incorporate sterols like CH in the bilayers [198]. Another approach has been the use of polymerized liposomes. Recently, polymerized liposomes were shown to maintain stability and retain at least 75 percent of their contents in the intestine after oral administration [199]. The third approach was to coat the outermost layer of liposomes with polymers. Unlike polymerized liposomes, polymer-coated liposomes are easy to prepare and do not raise toxicity concerns due to the fact that they are made of naturally-occurring phospholipids and polysaccharides. They have been shown to target tumor cells, including those in the brain [125], to provide lower leakage of drugs, and they may also interact with the epithelial lining of the GI tract increasing the residence time of the formulation. Recently, the potential of chitosan-coated liposomes containing insulin as a mucoadhesive dosage form to significantly lower blood glucose level in diabetic rats has been demonstrated [116]. Also, it has been shown that CH-pullulan reduced leakage of water-soluble Ara-C from liposomes and improved stability in simulated GI fluids [200]. Thus, liposomes may be made more stable in the GI tract as well as having greater residence times in the GI tract.

1.3. Rationale

Coating liposomes with polysaccharide derivatives is intended to mimic the cell wall of bacteria or plant cells. The cell walls are required to maintain the shape and rigidity of cells and to protect cell membrane against the chemical and physical stimuli, such as osmotic pressure and temperature changes. Hydrophobization of polysaccharide via attachment of a cholesterol moiety provides a stronger interaction between polymer and the bilayer. Such liposomes have exhibited slower release rate of drugs [125,200]. Since the ileum is assumed to have a more permeable epithelium [81-83] and the colon has lower proteolytic activity [86] combined with longer residence times of dosage forms (10-20h) than the upper GI tract [87], therefore stabilized liposomes with low leakage of contents should improve the possibilities of the oral delivery of insulin.in the lower GI tract. Adsorbed agents at liposome surfaces may also have the potential of enhancing interactions with enterocytes, thereby increasing GI residence time and absorption of entrapped solutes, such as insulin.

1.4. Aim

The overall aim of this research was to formulate a functionalized oral liposome delivery system of insulin as a model peptide drug.

1.5. Hypotheses

This research was based on the following hypotheses:

1. Liposomes formulated with insulin are stabilized by adsorbed hydrophobized polysaccharide with the result of decreased leakage in the GI tract.
2. Polymer-coated liposomes of insulin administered orally to diabetic rats predictably lower and maintain blood glucose levels.

1.6. Objectives

1. To prepare, purify and characterize cholesterol-derivatized polysaccharides;
2. To determine which liposome compositions and preparation methods yield high insulin encapsulation efficiencies while avoiding its degradation or inactivation;
3. To determine coating efficiencies (mg polymer associated with 100 mg lipid) of liposomes by hydrophobized polysaccharide (e.g. CHM) or CM-chitin as a function of polymer concenteration;
4. To characterize CHM-coated , CM-chitin-coated and uncoated liposomes with regard to: a) phase behavior and thermodynamic changes of the bilayer utilizing differential scanning calorimetry (DSC); b) changes in insulin encapsulation efficiency prior to and following polymer coating; c) size distribution; d) liposome integrity as a function of bile salt concentration at RT or 37 °C by measuring turbidity changes (i.e. dissolution of liposomes); e) insulin protection against proteases; f) insulin release from liposomes in buffer media, simulated gastric fluids (SGF), and simulated intestinal fluids (SIF);

5. To study blood glucose lowering in diabetic rats after intragastric administration of optimized liposome formulations, particularly comparing polymer-coated and uncoated liposomes.

2. EXPERIMENTAL

2.1. Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylcholine (DOPC), were obtained from Princeton Lipids, Princeton, NJ. Cholesterol (CH), stearylamine (SA), pullulan, mannan, dextran, and insulin were obtained from Sigma Chemical Co., St. Louis, MO and used as received. HPLC grade water and acetonitrile were obtained from Caledon Laboratories Ltd. and Fisher Chemicals, respectively. All other chemicals were reagent grade or better.

2.2. Methods

2.2.1. Polymer Derivatization

Cholesterol-derivatized polysaccharides were prepared in three steps according to a previously reported procedure [165], suitably modified to improve the derivatization ratio. Molecular structures of mannan, CHM, and intermediate products are depicted in Figure 2.2.

A. Carboxymethylation of polysaccharides (CM-PS). Carboxymethylation was achieved by reaction of 1.2 g polysaccharides, e.g. mannan, pullulan, or dextran with

0.12 g sodium hydride (NaH) in dry dimethyl sulfoxide (DMSO) for 4 h followed by reaction with 0.15 g bromomethyl acetate for 12h at room temperature (RT). Subsequently, the addition of absolute ethanol caused precipitation of the methylester derivative of carboxymethyl polysaccharide (CM-PS), which was then separated by filtration. The precipitate was washed with ethanol, then converted to CM-PS with stirring in a 0.2 M NaOH solution at RT for 4 h. Purification of the product was carried out by dialysis of the mixture against toluene-saturated water for 2 days and against MilliQ® water (Millipore®, Millford, MA) for 1 day. Infrared spectroscopy indicated the presence of carbonyl group at 1740 cm^{-1} as evidence of carboxymethylation (Figure 2.1).

B. Reaction of ethylendiamine with CM-PS: addition of an aminoethylcarbomethyl spacer on PS (AECM-PS). CM-PS (1 g) was reacted with ethylenediaminhydrochloride (3.2 g) at pH 4.7 for 12 h in the presence of 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (0.3 g) as a coupling agent to form aminoethylcarbomethyl - polysaccharide (AECM-PS) which was purified by dialysis against 0.2 M NaOH solution for 2 days and MilliQ® water for 1 day. Elemental analysis confirmed the substitution reaction by ethylenediamine (Table 2.1).

C. Addition of cholesterol to AECM-PS. AECM-PS was dissolved in dry DMSO and reacted with cholesterol chloroformate in dry DMF in the presence of pyridine. The mixture was stirred at 80 °C for 12 h and cooled to RT. Cholesterol-derivatized polysaccharide (CH-AECM-PS) produced was precipitated by absolute

ethanol and filtered. The precipitate was washed with ethanol and ether, dried under vacuum for 2 days, then stored in a refrigerator.

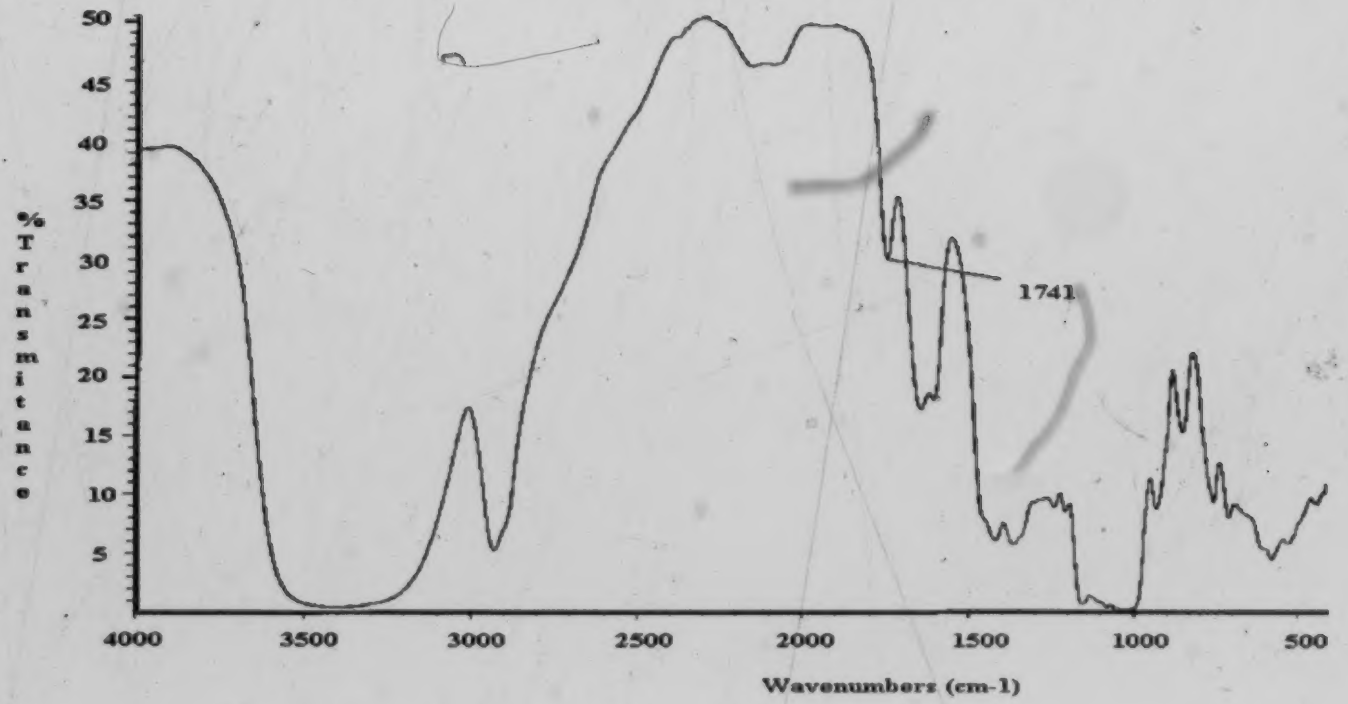
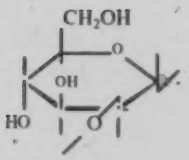
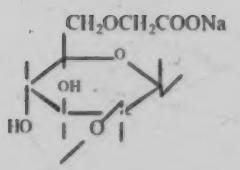


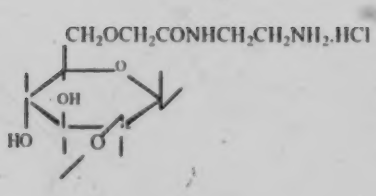
Figure 2.1. A typical IR spectrum of carboxymethyl polysaccharide (e.g. CM-mannan) in KBr. The presence of a carbonyl peak at 1741 cm^{-1} is evidence of carboxymethylation.



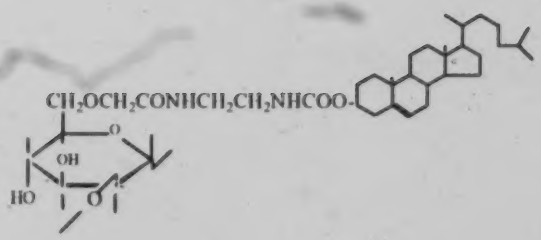
a) mannan



b) carboxymethyl-mannan



c) AECM-mannan



d) cholesterol-AECM-mannan (CHM)

Figure 2.2. Mannan and its derivatives

Table 2.1. Elemental analysis following ethylendiamine substitution of carboxymethylated polysaccharides

Batch[a]	Polysaccharide	ELEMENTAL ANALYSIS (%)		
		C	H	N
CHD1	Dextran	40.12	6.47	0.57
CHP0.4	Pullulan	40.57	5.96	0.56
CHP1	Pullulan	42.47	6.45	0.60
CHP4.2	Pullulan	40.74	6.25	1.37
Pure Mannan	Mannan	39.84	6.16	0.26
CHM0.1	Mannan	40.06	6.04	0.26
CHM2	Mannan	42.49	6.49	1.43
CHM2.2	Mannan	40.31	6.05	2.27
CHM2.3	Mannan	39.56	5.80	2.25

[a] CHD, CHP, and CHM correspond to cholestrol-dextran, cholestrol-pullulan, and cholestrol-mannan, respectively. The number of cholesterol moieties per 100 saccharide units is indicated by the number attached.

2.2.2. Analytical

2.2.2.1. HPLC analysis of insulin

Insulin analysis was performed by gradient controlled HPLC equipped with a UV-visible detector (Waters®, Division of. Millipore® Corporation, Milford, MA). A reversed phase C18, 10 µm 3.9 x 300 mm µBondapack column (Millipore®) was used. The gradient system consisted of mobile phase A, 0.055% trifluoroacetic acid (TFA) in water, and mobile phase B, 0.050% TFA in acetonitrile (ACN). A linear gradient was run from 25 to 40 percent mobile phase B within 15 minutes, maintained at 40 percent mobile phase B for 8 min, then returned to the initial concentration (25/75, B/A ratio) and maintained for 15 min prior to the next injection. The flow rate was 1 ml/min.

2.2.2.2. Determination of insulin adsorption

All glassware was siliconized with dichlorodimethylsilane (DCDMS) or Surfasil® (consisting primarily of dichlorooctamethyltetrasiloxane). Insulin solutions at various concentrations were placed in polypropylene tubes, as well as uncoated, Surfasil-coated, and DCDMS-coated glass tubes and left at RT for 1 h. The extent of insulin adsorption was determined from the areas under the insulin concentration peaks.

2.2.3. Liposome Preparation

2.2.3.1. Classic film method

Phospholipids such as DPPC or DSPC together with CH and a charged adduct like SA or DMPG dissolved in chloroform were combined in specified molar proportions to give a final lipid concentration of 15 mM. Rotary evaporation of the solvent under reduced pressure at 50 °C produced a thin film in the round bottom flask. The flask was flushed with N_2, then stored overnight in a vacuum desiccator at 40°C. Insulin was dissolved in 0.01 or 0.05 M HCl, the solution was neutralized by addition of a small volume of 1 M NaOH, then diluted with 10 mM phosphate buffer saline pH 7.4 to give the desired concentration of insulin. Subsequently, the lipid film was hydrated with the insulin solution to give a final drug to lipid weight ratio of 0.5-1 to 10. Dispersion and swelling of the lipid were carried out by using glass beads at above the phospholipid phase transition temperature for 5 min, followed by storage at RT for 3 h. Liposomes were bath-sonicated (Heat Systems-Ultrasonic, Inc., Plainview, NY) for 10 min at 4°C and subsequently stored at RT for 1 h. Liposomes were incubated with an equal volume of 0.5% carboxymethyl chitin (CM-chitin) or 1% cholesterol-derivatized mannan (CHM) solutions in 10 mM phosphate bufferred saline solution (PBS) at pH 7.4 to form polymer-coated liposomes and stored in the refrigerator overnight. The weight ratio of polymer:lipid was varied over the range of 0.1-1 depending on requirements. Uncoated and polymer-coated liposomes were centrifuged at 10,000-130,000 x g for 15-30 min at 4 °C (Beckman Model L8-55 Ultracentrifuge). The supernatant containing free insulin

and/or free polymer was removed and the pellet was resuspended in PBS solution. This procedure was repeated twice.

2.2.3.2. Dehydration-rehydration (DRV) method

Liposomes were prepared according to Kirby & Gregoriadis (1984) with some modifications. Briefly, 15 mM lipid solution in chloroform was dried in a round-bottom flask by rotary evaporation. The flask was flushed with N_2, then stored overnight in a vacuum desiccator at 40°C. The lipid film was hydrated with water above the phospholipid T_m for 5 min, followed by probe-sonication for 1 min at RT. Liposomes were centrifuged at 5000 x g to separate titanium particles released from the sonicator probe. The supernatant containing SUVs was freeze-dried and a small volume (~0.4 ml/100 mg lipid) of insulin solution in 10 mM PBS (16 mg/ml) was used to rehydrate the lyophilized SUVs. After 30 min liposomes were warmed > T_m for 5 min in the rotary evaporator. After cooling to RT liposomes were diluted with 10 mM PBS at pH 7.4 and left for 3 h at RT. In one experiment an insulin solution was prepared in 5 mM phosphate bufferred solution at pH 7.4 and added to SUVs before freeze-drying. Lyophilized liposomes were then rehydrated with 10 mM PBS pH 7.4 solution and treated as before. Polymer-coated liposomes were prepared as previously described (section 2.2.3.1). Uncoated and polymer-coated liposomes were ultracentrifuged as before. The supernatant containing free insulin and/or free polymer was removed and the pellet was resuspended. This procedure was repeated twice.

2.2.4. Liposome Characterization

2.2.4.1. Determination of polymer coating efficiency

Determination of the amount of polymer which coated a liposome preparation was carried out by modification of a procedure which was reported previously for a polysaccharide [201]. Briefly, the pellets of polymer-coated liposomes which were obtained after two washings were resuspended in PBS, then mixed with 4 ml of a solution of 25 mM cetyltrimethylammonium bromide (CTAB):isopropanol, 9:1 v/v. The lipid concentration was about 1 mM. The samples were subjected to heating > T_m for 5 min, then equilibrated at RT for 15 min. The turbidity of the colloidal suspension was measured spectrophotometrically at 400 nm and polymer concentrations were determined from a standard curve. Absorbances were corrected for the presence of lipid after treating uncoated liposomes in a similar fashion. Coating efficiency (CE) was calculated as a weight ratio of polymer to lipid (percent) [201].

2.2.4.2. Encapsulation efficiency

The pellets obtained from centrifugation after two washings (see sections 2.2.3.1 and 2.2.3.2) were resuspended in 10 mM PBS. The influence of detergent on liposome solubilization was determined by dissolving 300 µl of liposome suspension with 200 µl of 50 mM CTAB, 5% Triton X-100, or 5% SDS. Each detergent solution also contained 25 µg/ml phenol (as an internal HPLC standard). The mixture was then heated > T_m for 5 min. The clear solution was quantitatively analyzed for insulin by HPLC (see 2.2.3.1).

2.2.4.3. Particle size measurement

Samples of liposomes (50 µl) were diluted to 3 ml with PBS solution, then analyzed by dynamic laser light scattering (Brookhaven Instruments BI-90 Particle sizer, Holtsville, New York) at RT. The laser power is 5 mW, HeNe, vertically polarized. This instrument can nominally measures in a size range of 5 nm - 5 µm depending on concentration and relative light scattering power with an accuracy of ±1-2% for monodisperse samples and a repeatability of ±1-2% with dust free samples.

2.2.4.4. Differential scanning calorimetry (DSC)

Calorimetric measurements were performed with a Seiko thermal analyzer, model SSC/5200 (Seiko Instruments Inc, USA). The system was configured for data analysis using a Hewlett Packard® workstation model 712. Indium, palmitic acid, and benzoic acid were used to calibrate the instrument.

Empty or insulin-encapsulated liposomes were prepared from lipid solutions of DPPC, DPPC:CH (7:2 m.r.), DPPC:SA (7:1 m.r.), DPPC:DMPG (7:0.5 m.r.) in chloroform as previously described (see 2.2.2.1. and 2.2.2.2.). Polymer-coated liposomes were prepared by addition of 0.5% CM-chitin or 1% CHM solutions in pH 7.4 PBS to empty liposomes (hydrated with buffer only). After removal of free insulin and/or free polymer, the liposomal pellets were resuspended in 3 ml PBS, divided into equal volumes in microcentrifuge tubes, then centrifuged at 14,000 rpm at RT for 5 min. The supernatants were discarded and the pellets were resuspended in 50 µl PBS and transferred to 100 µl aluminum crucibles for analysis. A sealed pan containing 50 µl PBS

solution was used as a reference. The samples were scanned at a rate of 1°C.min^{-1} from 23 - 75 °C.

2.2.5. Liposome Behavior in Various Media

2.2.5.1. Stability in sodium cholate solutions

The stability of liposomes was assessed from turbidities of their suspensions in sodium cholate (SC) solutions. Uncoated or polymer-coated liposomes were prepared, washed and resuspended in PBS solution as before. One ml liposome suspension was diluted to 10 ml using PBS or various cocncentrations of SC solutions. Turbidities were defined as absorbances measured spectrophotometrically at 400 nm [201]. Samples were allowed to equilibrate for 15 min (no sample exhibited a turbidity change after 60 min, see Figure 3.18 and 3.20) before a reading was made. [202]Initial absorbances were considered as 100 percent turbidity.

2.2.5.2. Stability of insulin in liposomes in the presence of α-chymotrypsin

The protection of insulin encapsulated in liposomes was evaluated in α-chymotrypsin solution at pH 7.8 (optimal pH for enzyme activity) and 37 °C. Liposomes were prepared by the classic film method as before. The liposomal pellet was resuspended in pH 7.8 phosphate buffered solution to which α-chymotrypsin had been added. α-Chymotrypsin was also added to insulin solution (as control). The final concentrations of lipid and the enzyme were 5 mM and 0.5 μM, respectively. Samples were removed at various time

intervals, centrifuged at 14,000 rpm for 5 min, then 0.5 ml 0.05% TFA was added to the pellets to inactivate traces of enzyme present at the liposome surfaces. Subsequently, 0.5 ml of 40 mM CTAB and 20 μg/ml phenol were added to each tube and heated > T_m for 5 min. The solution obtained was centrifuged as before to separate the polymer. The insulin concentration was analyzed by HPLC and the percent insulin remaining intact was calculated.

2.2.5.3. Release of insulin

2.2.5.3.1. In buffer solution

The rates of insulin release from uncoated or polymer-coated liposomes were determined at pH 2 or pH 7.4 at 37^{O}C (Dubnoff Metabolic Shaker). At various time intervals liposome samples stored in microcentrifuge tubes were centrifuged at 14,000 rpm for 5 min, the supernatants removed and the pellets diluted with 10 mM PBS solution at pH 7.4 containing 20 mM CTAB and 10 μg/ml phenol at a fixed lipid concentration of 1 mM. After heating > T_m for 5 min the solution was centrifuged at 14,000 rpm for 5 min. The fraction of insulin released from liposomes was determined from

$$\% \text{ Insulin Released} = [1 - (C_p / C_{po})] \times 100 \qquad (1)$$

where C_p is the concentration in the pellet at any time, t, and C_{po} is the initial drug concentration in the pellet before incubation.

2.2.5.3.2. In simulated gastric and intestinal fluids

Polymer-coated or uncoated liposomes were prepared as before but containing ^{125}I-insulin. Liposomal pellets were resuspended in simulated gastric (SGF) or simulated intestinal (SIF) fluids, USP, divided into 1 ml samples in microcentrifuge tubes and incubated at 37^{O}C (Dubnoff Metabolic Shaker). At various time intervals samples were centrifuged at 14,000 rpm for 5 min, the supernatant removed from the pellet and γ–counting performed on both fractions. The fraction of ^{125}I-insulin released from liposomes was determined from

$$\% \text{ Released} = (C-C_0) \times 100/(Ct-C_0) \quad (2)$$

where C_0 is the initial drug concentration in the supernatant before incubation, C_t is the total drug concentration in the liposomes and C is the concentration in the supernatant at time, t [157].

2.2.6. In Vivo Studies

2.2.6.1. Animals

Male Wistar Furth rats (200-250g) were bought from Harlan Sprague Dawly® (Indianapolis, Indiana) and housed in a light- and temperature-controlled environment.

Urine was collected and its volume was recorded daily. Food and water consumption, and weight of the rats were also monitored on a regular basis.

2.2.6.2. Induction of diabetes mellitus

Animals were anesthetized by Halothane®. To induce diabetes mellitus, rats were injected with 55 mg/kg of streptozocin (STZ) in citrate buffer (pH 4.5) via the penial vein. The buffer had been sterilized by filtration (0.22 μm filter membranes). Blood glucose levels were measured before and on days 7, 8 and 9 after STZ injection using reagent test strips with a Blood Glucose Sensor Electrodes apparatus (Medisense®, England). Rats were considered diabetic and included in the study when serum glucose levels were greater than 300 mg/dl.

2.2.6.3. Dosing and sampling

The study was performed on day 10 following STZ injection after an overnight fast of 16 h. Water was allowed *ad libitum*. Diabetic rats were intragastrically administered either insulin solution, empty liposomes, PBS solution, or insulin liposomes. Insulin liposomes consisted of DSPC:CH:X (7:2:X m.r., where X represents SA or DMPG mole ratio) were prepared by the DRV method and after characterization for insulin content were administrated to animals as described previously. Prior to and at specified time intervals over a 12 h period blood samples (~100 μl) were collected by tail vein puncture and collected in heparinized capillary tubes. The samples were centrifuged for 5 min at RT and the plasma were collected and frozen for further glucose analysis.

2.2.6.4. Glucose assay

The plasma samples were diluted with normal saline. Blood glucose was measured by the glucose oxygenase method [203] using an Astra-8® analyzer (Beckman® Instruments Inc., USA). Each sample was measured twice and averaged.

2.2.6.5. Treatment of data

Blood glucose concentrations were determined twice prior to dosing and the mean concentration was considered as the 100 percent level. All following concentration-time data were expressed as a fraction of the base line, considering the fact that blood glucose concentrations over 12 h following intragastric administeration of PBS, empty liposomes, or insulin solution to diabetic rats (control groups) were not significantly different from the base line (assuming a flat base line). The mean ± S.E. of each concentration-time point in each treatment group was calculated. Comparisons between control groups, and between a control group (insulin solution) and other treatment groups were performed using a one-way analysis of variance (ANOVA) followed by a multiple comparison test. The Sigmastat® version 2.0 was used to carry out statistical tests.

2.2.6.6. Pharmacodynamic analysis

The mean of percent blood glucose concentrations at each time point was subtracted from 100 percent and the areas above the percent blood glucose-time curve ($AAC_{0\text{-}12\ h}$) were estimated by the trapezoidal rule as reported by Touitou and Rubinstein [204].

3. RESULTS

3.1. Polymer Derivatization

Derivatization of polysaccharide with cholesterol was carried out in three stages. The degree of ethylendiamine substitution on carboxymethylated polysaccharide (formation of AECM spacer) was determined by elemental analysis (stage 2). The degree of substitution of CH (stage 3) was estimated from ^{1}H-NMR spectra, where methyl and methylene protons of CH molecules produced signals between 0.2 and 2.4 ppm (Figure 3.1). Such signals were absent in the ^{1}H-NMR spectra of the polysaccharides. The number of molecules of AECM spacer or cholesterol derivatized per 100 saccharide units were calculated. Details of the polymer derivitization are summarized in Table 3.1.

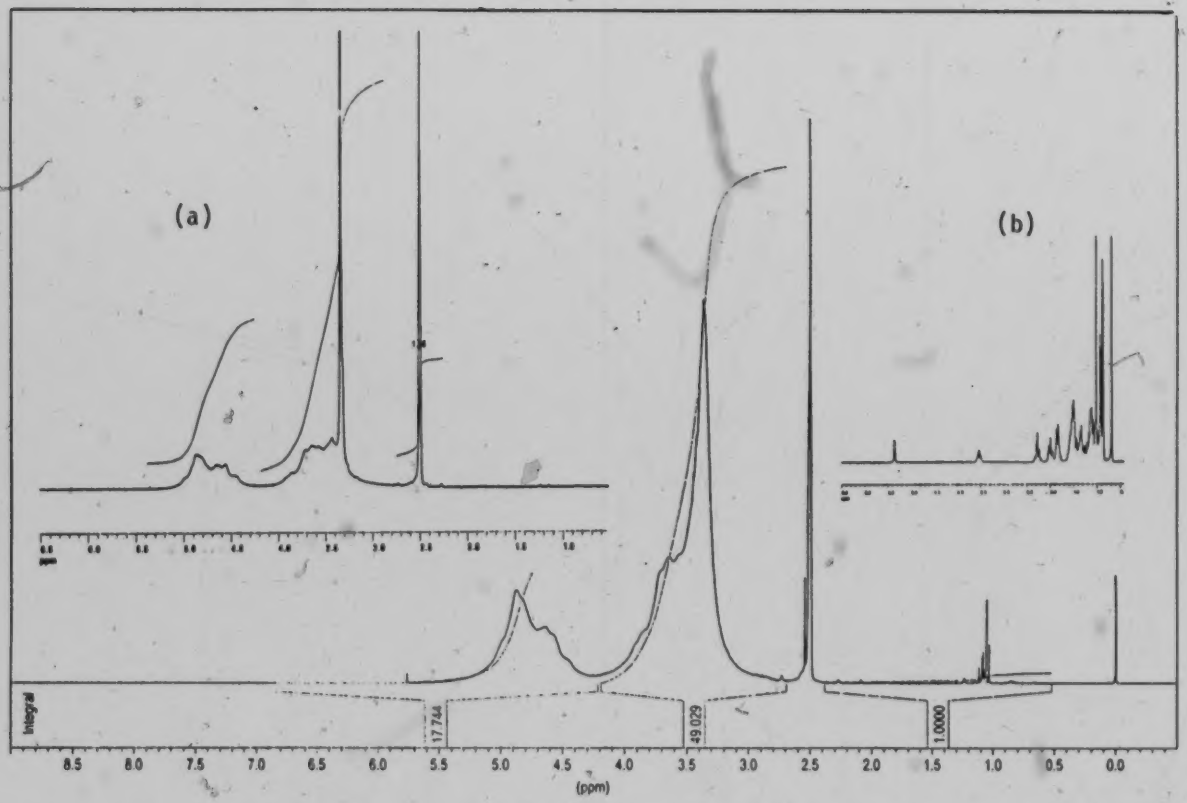


Figure 3.1. ^{1}H-NMR spectra of cholesterol-derivatized mannan (CHM) in DMSO-d_6 at RT. Inset (a) and (b) are mannan and cholesterol spectra, respectively. Protons of mannan from CHM appeared at 2.6-6.6 ppm and of cholesterol at 0.2-2.4 ppm as evidence of derivatization.

Table 3.1. Summary of polysaccharides derivatization details

Batch[a]	Polysaccharide	AECM%[b]	Cholesterol%[c]
CHD1.5	Dextran	3.6	1.5
CHD1	Dextran	1.1	1.0
CHP4.2	Pullulan	4.3	4.2
CHP0.4	Pullulan	1.2	0.4
CHM2.3	Mannan	8.8	2.3
CHM2.2	Mannan	8.4	2.3
CHM2	Mannan	4.3	1.9
CHM1.2	Mannan	n.d.	1.2
CHM1	Mannan	n.d.	1.0
CHM0.8	Mannan	n.d.	0.8
CHM0.3	Mannan	n.d.	0.3

a. CHD, CHP, and CHM correspond to cholesterol-derivatized- dextran, pullulan, and mannan, respectively. The number of cholesterol moieties per 100 saccharide units is indicated by the number attached.

b. aminoethylcarbamethyl, representing the number of molecules of AECM spacer per 100 saccharide units determined from elemental analysis (Table 2.1).

c. Cholesterol% represents the number of molecules of cholesterol per 100 saccharide units estimated from ^{1}H-NMR spectra.

3.2. Analytical

3.2.1. Insulin Assay

The HPLC procedure of gradient elution allowed separation of an insulin peak from its desamido derivative, over a narrow range of acetonitrile concentrations (Figure 3.2). Solutions of various concentrations of insulin spiked with 10 µg/ml phenol as internal standard in 10 mM pH 7.4 PBS were used for standard curves. A linear correlation of insulin/phenol peak area ratio as a function of insulin concentration over a 0-300 µg/ml range was obtained. Figure 3.3 reveals a typical standard curve of insulin in the concentration range often used. Correlation coefficients generally exceeded 0.99. The minimum detectable amount of insulin was about 20 ng, however, quantification was generally achieved for greater than 200 ng insulin.

3.2.2. Insulin Adsorption

A study of the adsorption of insulin at various surfaces as a function of concentration yielded the results depicted in Figure 3.4. The loss of insulin was more pronounced at lower concentrations where it was stored in uncoated glass tubes. The minimum adsorption of insulin was obtained with Surfasil®-coated glass tubes. Polypropylene tubes were as good at preventing drug adsorption under conditions of the experiment.

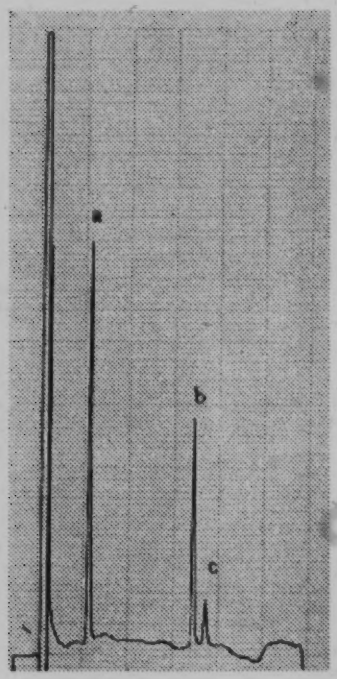


Figure 3.2. HPLC chromatograms of (a) phenol, (b) insulin, (c) desamido-insulin obtained by a gradient control method consisting of mobile phase A, 0.055% trifluoroacetic acid (TFA) in water, and mobile phase B, 0.050% TFA in acetonitrile. A linear gradient was run from 25 to 40 percent mobile phase B within 15 min, maintained at 40 percent mobile phase B for 8 min, then returned to the initial concentration (25/75, B/A ratio) and maintained for 15 min prior to the next injection. Flow rate, constant at 1 ml/min; detection, UV at 210 nm; column, C18 μBondapack (Millipore®).

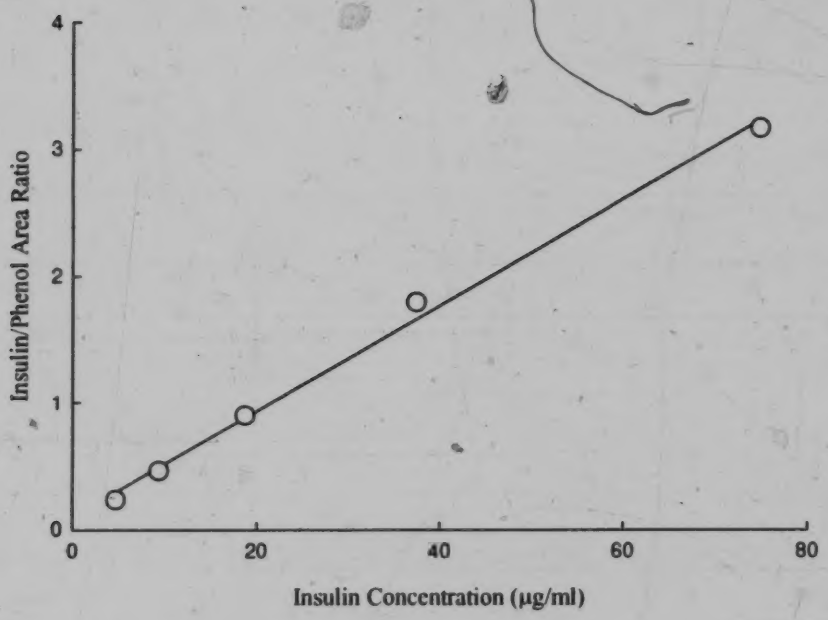


Fig 3.3. A typical standard curve of insulin determined by a gradient control HPLC method (see details in Fig 3.2), n=3, $r^2 > 0.99$.

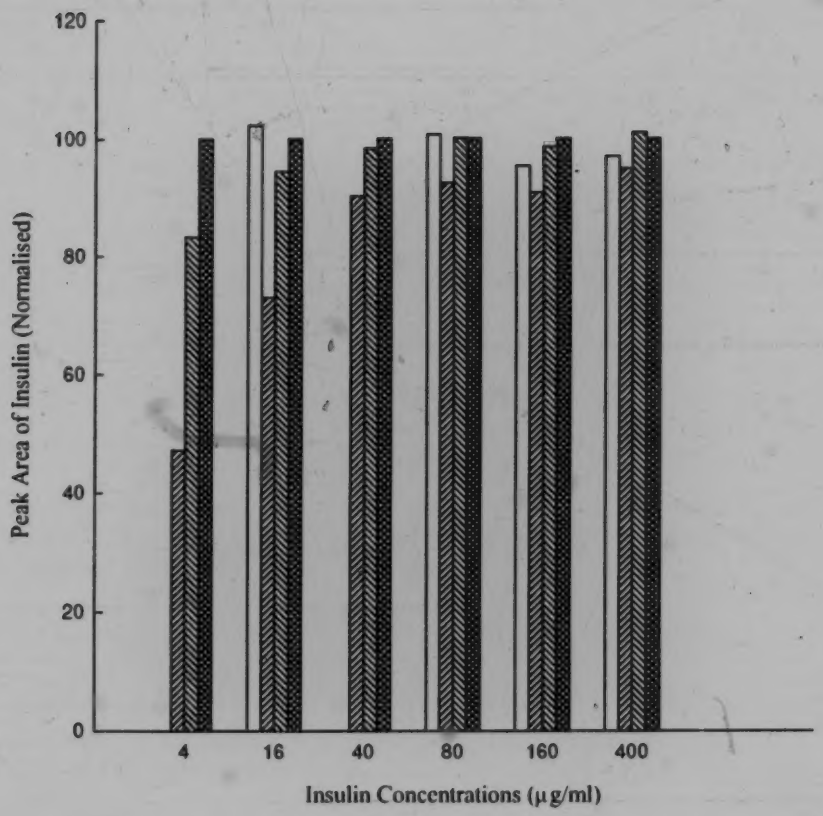


Figure 3.4. Comparative adsorption of insulin on various tube surfaces after 1h incubation at RT. ▭, polypropylene; ▨, uncoated glass; ▩, dimethyldichlorosilane-; and ■, Surfasil-coated glass tubes. The peak areas of insulin at each concentration were normalized based on the value obtained from Surfasil-coated glass tubes which generally gave the highest responses

3.3. Liposome Characterization

3.3.1. Coating Efficiency of Liposomes by CM-chitin and CHM

The standard curves of polymer turbidities in a 20 mM pH 7.4 CTAB:isopropanol (9:1 v/v) solution are shown in Figure 3.5. Linear correlations between turbidities and concentrations were obtained for CM-chitin (0-40 μg/ml) and CHM (0-320 μg/ml). The turbidities were stable for long periods of time being not significantly changed after 60 min. The coating efficiency (CE) of liposomes as a function of polymer concentration is depicted in Figure 3.6. Increasing CM-chitin concentration up to 0.5% increased the CE to about 3%. No further increase in CE was observed when a CM-chitin concentration of 1% was used (Figure 3.6a.). In contrast, a higher CE of about 10% was obtained when liposomes were coated from 1% CHM solutions.

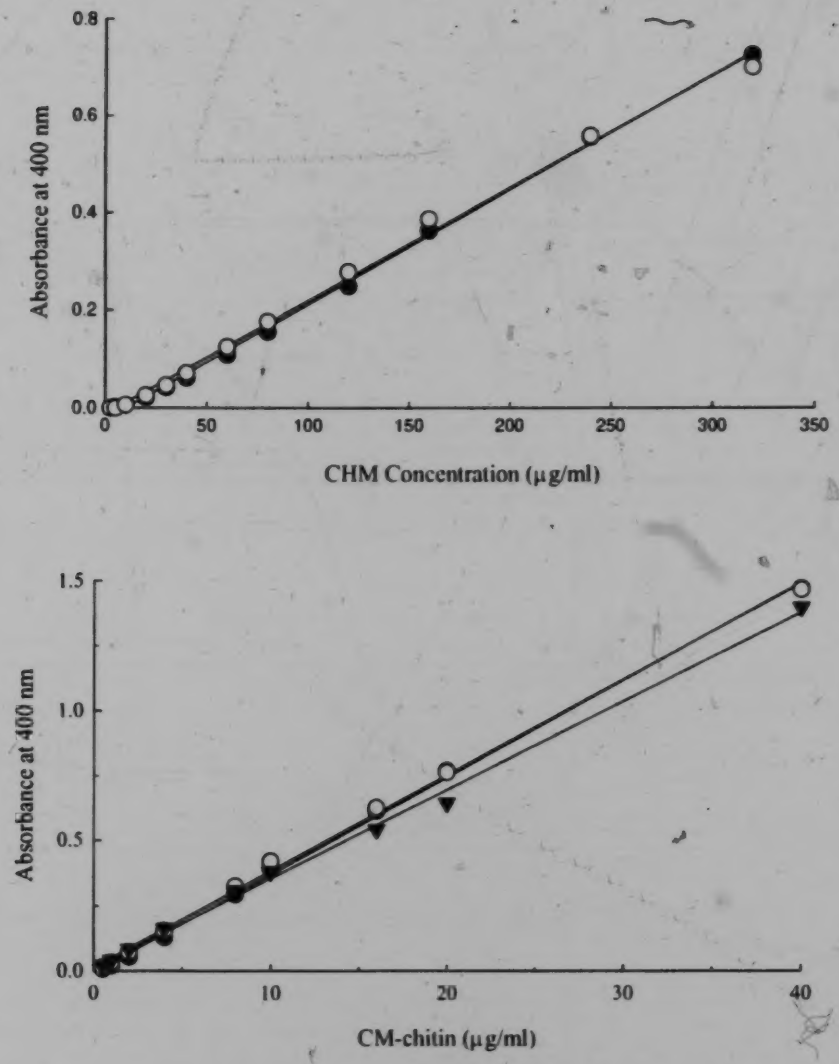


Figure 3.5. Turbidity standard curves of CHM (upper) and CM-chitin (lower) dispersions in a 20 mM CTAB (pH 7.4):isopropanol (9:1 v/v) solution determined at 400 nm after (●) 15 min, (O) 60 min and (▼) 16 h incubation. Values represent means of 2 or 3.

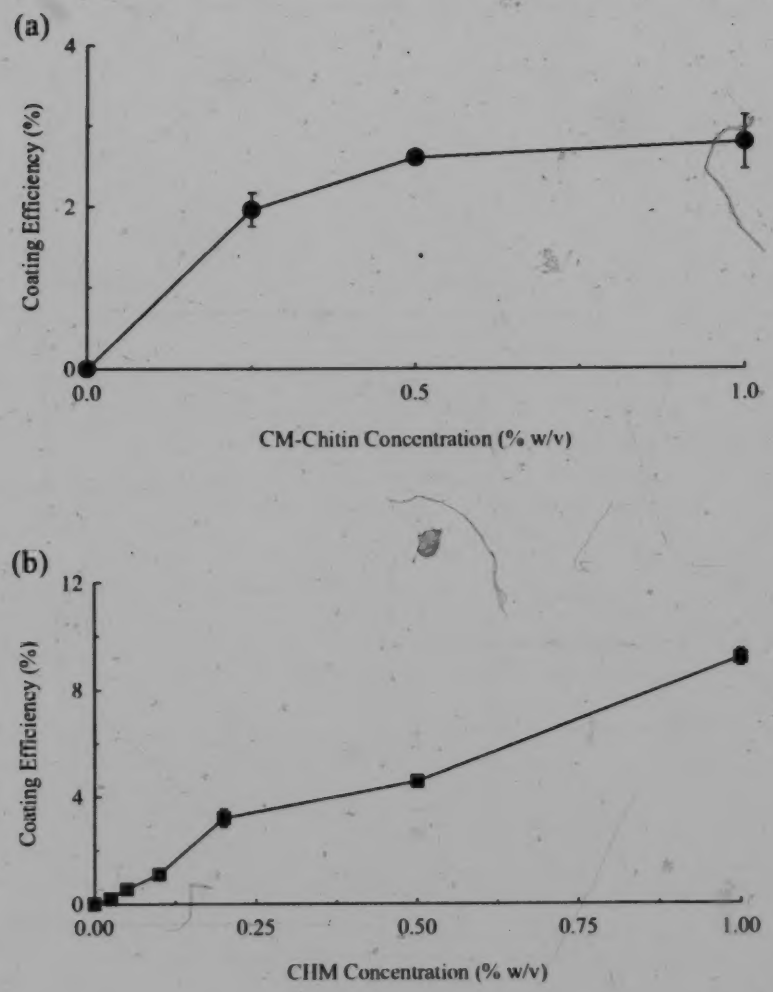


Figure 3.6. Coating efficiency of (a) DPPC:CH:SA (7:2:1 m.r.) liposomes, and (b) DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes as a function of polymer concentration at pH 7.4. Coating efficiency is defined as mg polymer associated with 100 mg lipid. Means ± SE, n=3.

3.3.2. Insulin Encapsulation Efficiency (EE) and Loading Efficiency (LE)

The effects of Triton X-100, CTAB, and SDS on determination of liposomal insulin are depicted in Figure 3.7. Triton X-100 interfered with insulin analysis by HPLC and could not be used. Liposomes composed of DPPC:CH:SA were more effectively disrupted by CTAB than by SDS as was evident from the measurement of the insulin content of liposomal pellets (Figure 3.7).

The EE of insulin in positively-charged liposome (PCL) composed of DPPC:CH:SA (7:2:X m.r.) prepared by the CF method increased as the mole ratio of SA increased (Figure 3.7). A maximum EE of 67 percent was obtained at a SA m.r. of 2, compared to a low 2.25 percent EE obtained with neutral liposomes (i.e. no SA). Negatively-charged liposomes (NCL) composed of DPPC:CH:DMPG (7:2:X m.r.) prepared by the CF method yielded a maximum EE of 21.9 percent at a DMPG m.r. of 0.5 (Figure 3.8). The EE as a function of volume of insulin solution is depicted in Figure 3.9. It is apparent that small volumes used to prepare liposomes were more effective than large volumes, each containing the same amount of insulin.

The EE and loading of insulin in liposomes as a function of preparation technique or liposome coating are shown in Table 3.2. Generally, the DRV technique of liposome preparation yielded higher EEs and loadings, compared to the CF method, particularly with NCLs (EE of 45.6 versus 21.9 percent by CF). The EE of insulin in PCLs was higher (about 51 percent) approximately the same using either the CF or DRV methods. Coating liposomes with CM-Chitin resulted in a lowering of the EE compared to uncoated

vesicles (Table 3.2), whereas liposomes coated with CHM resulted in a higher EE than uncoated liposomes (Table 3.2 and Figure 3.10.).

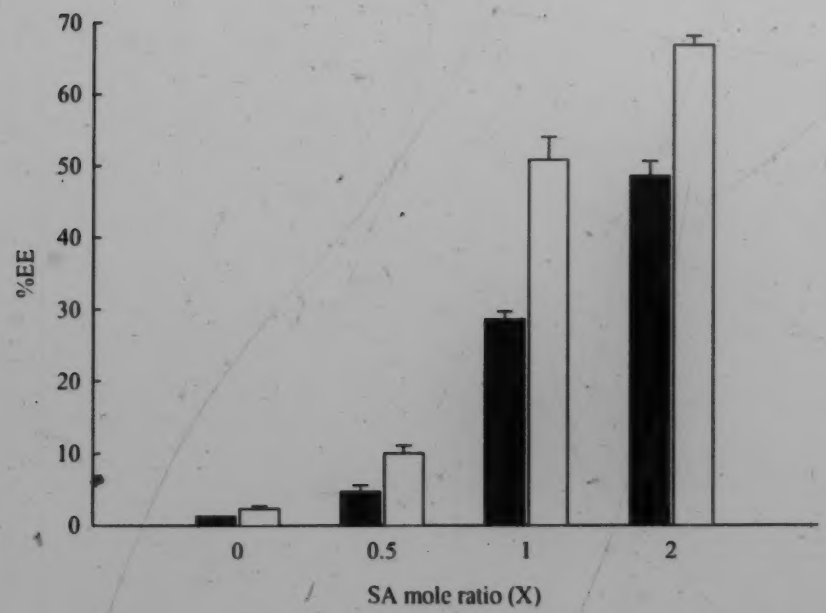


Figure 3.7. The effects of detergents on the assessment of EE of insulin in DPPC:CH:SA (7:2:X m.r.) liposomes. Insulin was measured by HPLC following liposome disruption in a solution of 5% SDS, ■; or 20 mM CTAB, □. Final lipid concentrations were 0.5 mM. Liposomes treated with detergents were heated > T_m of DPPC for 5 min. Means ± SE, n=3.

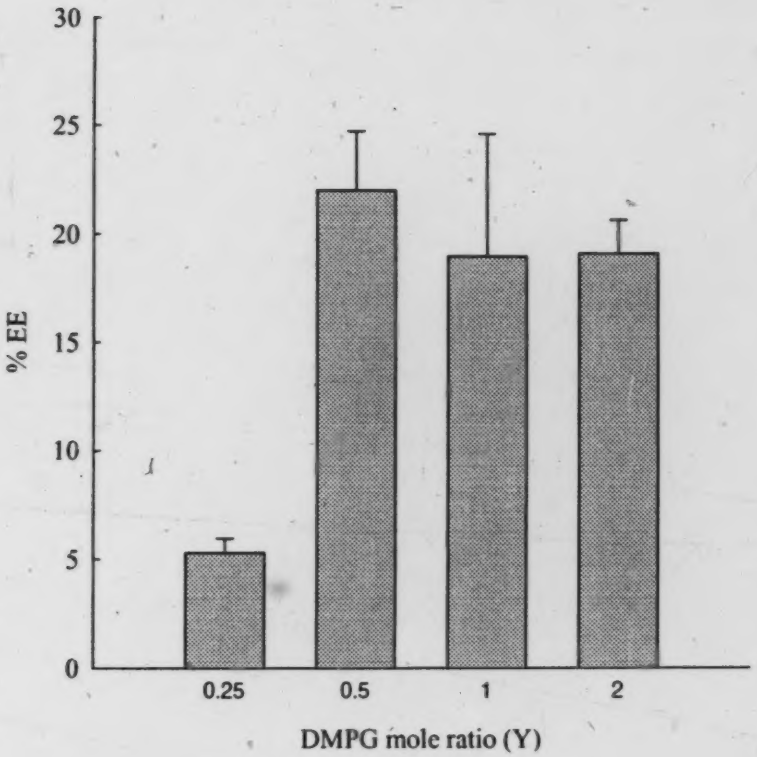


Figure 3.8. EE of insulin by DPPC:CH:DMPG (7:2:Y m.r.) liposomes prepared by the CF method following pellet disruption by 20 mM CTAB. Final lipid concentrations were 0.5 mM. Liposomes treated with detergents were heated above T_m of DPPC for 5 min. Means ± SE, n=3.

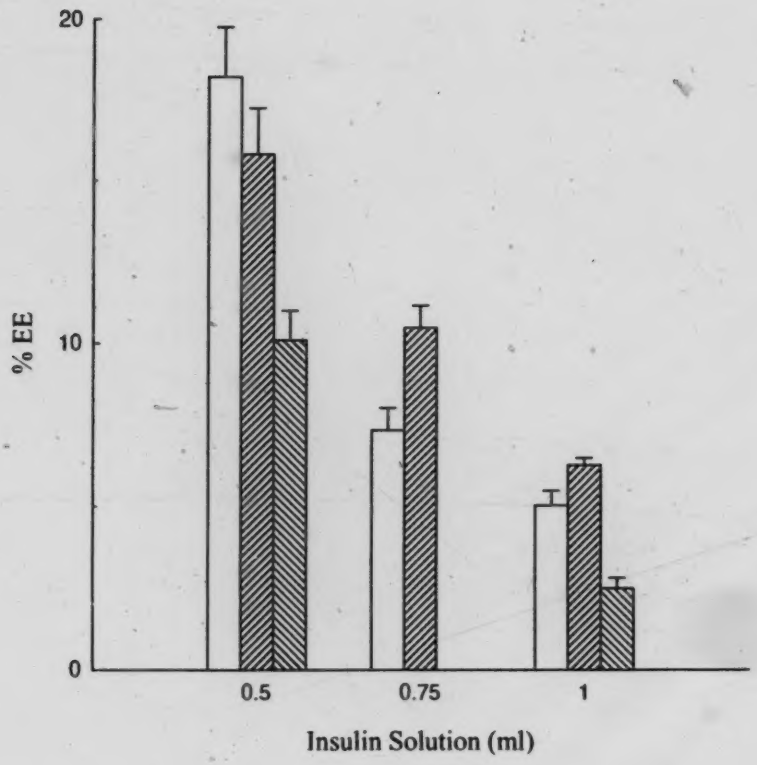


Figure 3.9. Effect of the volume of insulin solution on insulin EE in NCL of various compositions prepared by the CF method. All solutions contained the same amount of insulin. ▭, DPPC:CH:DPPG (7:1:0.5 m.r.); ▨, DPPC:CH:DPPG (7:2:0.5 m.r.); ▨, DOPC:DPPG (7:0.5 m.r.). Means ± SE (n=3).

Table 3.2. Summary of insulin EE and loading in PCL and NCL as functions of preparation techniques and polymer coating[a,b]

Coating Status	Preparation Technique	PCL[c]		NCL[d]	
		%EE[e]	%Loading[f]	%EE	%Loading
Uncoated	CF[g]	50.7 ± 3.2	2.5 ± 0.16	21.9 ± 1.27	2.2 ± 0.27
	DRV[h]	51.2 ± 0.69	5.2 ± 0.07	45.6 ± 0.95	4.56 ± 0.09
CHM-coated[i]	CF	n.d.[j]	n.d.	34.2 ± 1.6	3.3 ± 0.14
	DRV	68 ± 3.3	6.9 ± 0.34	71.6 ± 2.3	7.1 ± 0.19
CM-chitin-coated[k]	CF	43.8 ± 2.71	2.2 ± 0.13	n.d.	n.d.

a. Means ± SE, n=3

b. Initial drug/lipid wt ratios used to prepare liposome were 1/10 except for PCL prepared by CF which was 0.5/10.

c. DPPC:CH:SA (7:2:1 m.r.) liposomes

d. DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes

e. percent of insulin (of initial amount) encapsulated in liposomes

f. mg of insulin per 100 mg lipid

g. classic film method

h. dehydration-rehydration vesicles

i. from 1% CHM solution

j. not determined

k. from 0.5% CM-chitin solution.

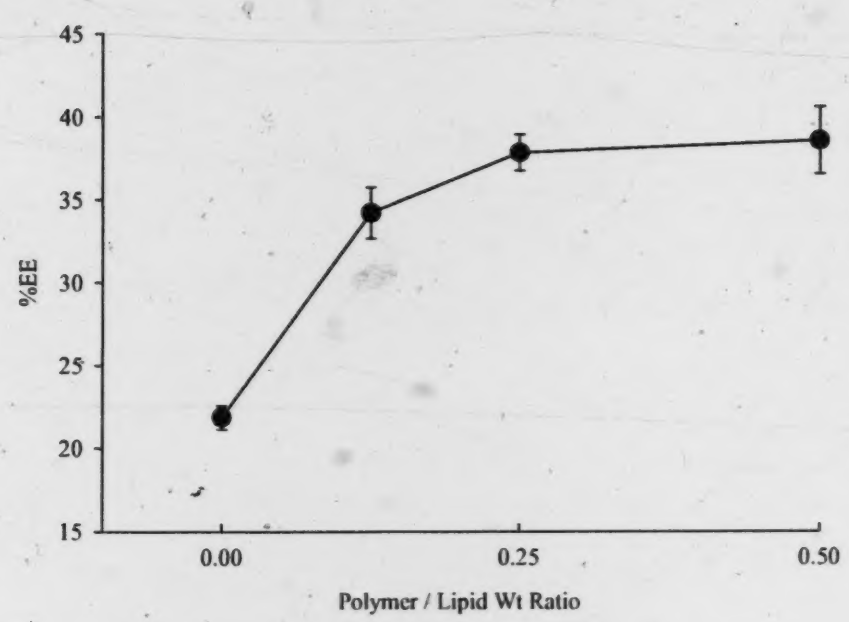


Figure 3.10. Effects of CHM-coating on insulin EE in NCL composed of DPPC:CH:DMPG (7:2:0.5 m.r.) prepared by the CF method. Means ± SE, n=3.

3.3.3. Liposome Size Determination

Liposomes had log-normal size distributions. Examples of such plots are depicted in Figures 3.11 and 3.12. Increasing the mole ratio of charged adducts, i.e. SA or DMPG or DPPG, resulted in decreasing sizes of liposomes (Figures 3.13 and 3.14). A summary of the size study of liposomes as functions of composition, preparation technique, and coating status is presented in Table 3.3. Neutral liposomes were generally larger than charged liposomes. The sizes of PCL prepared by the CF method were larger than the same liposomes prepared by the DRV method. In contrast, NCL prepared by the DRV were smaller than those prepared by the CF method. The sizes of neutral liposomes coated by CHM or CM-chitin was not different from the same uncoated liposomes. Coating NCL by CHM did not yield a change in size whereas CM-chitin-coated PCL were larger than uncoated-PCL.

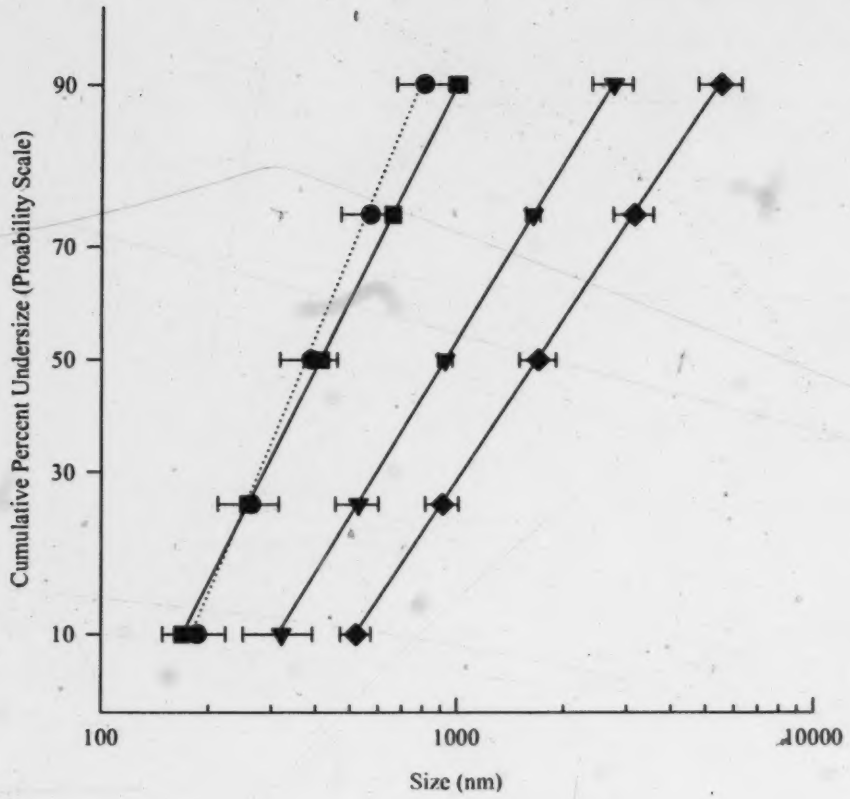


Figure 3.11. Log-probability plots of sizes of liposomes composed of DPPC:CH:DPPG (7:2:X m.r.) measured by dynamic laser light scattering (Brookhaven Instruments BI-90 Particle Sizer, Holtsville, New York), where x denotes mole ratio of DPPG as ●, 2; ■,1; ▼, 0.5; and ◆, 0.25.

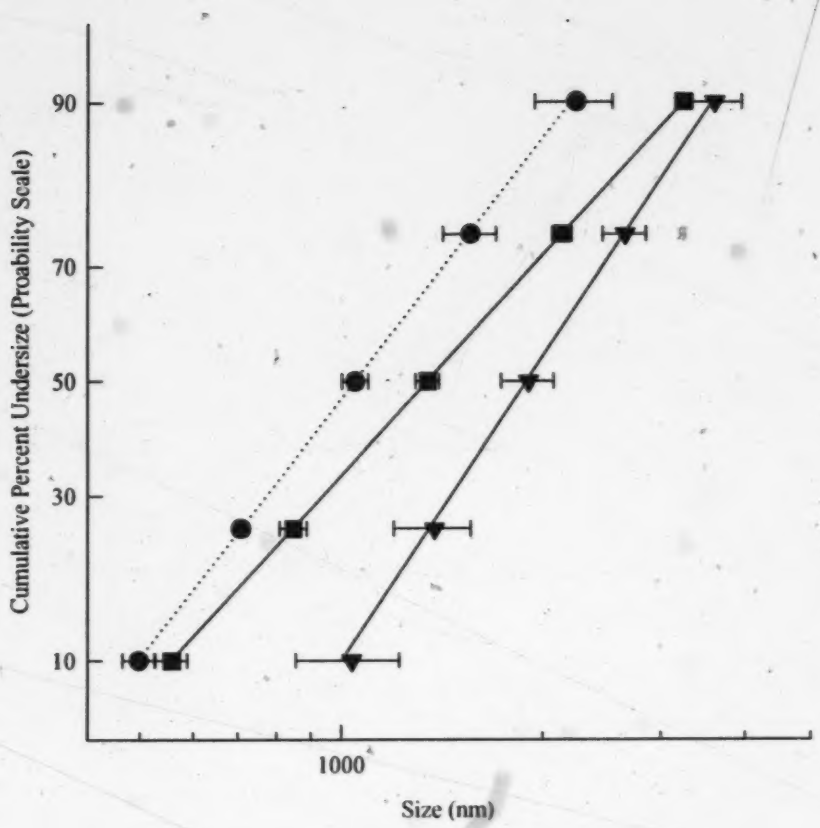


Figure 3.12. An example of log-probability plots of sizes of liposomes composed of DPPC:CH:DMPG (7:2:X m.r.) measured by dynamic laser light scattering (Brookhaven Instruments BI-90 Particle Sizer), where x denotes mole ratio of DMPG as ●, 2; ■,1; and ▼, 0.5.

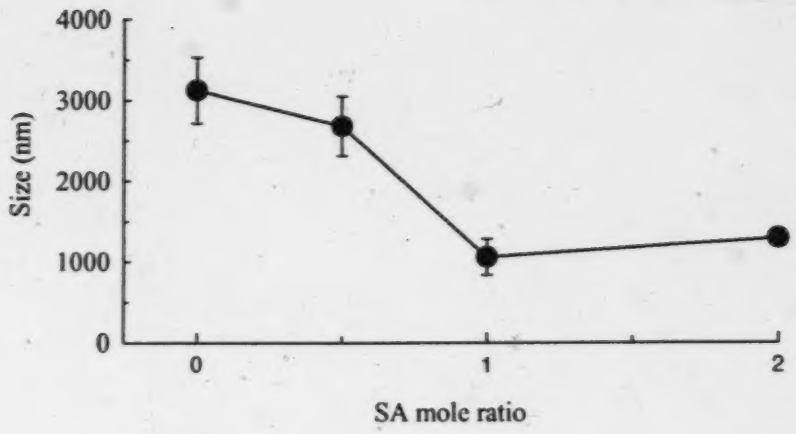


Figure 3.13. Effect of SA mole ratio on the size of DPPC:CH(7:2 m.r.) liposomes prepared by the CF method. Means ± SE, n=3.

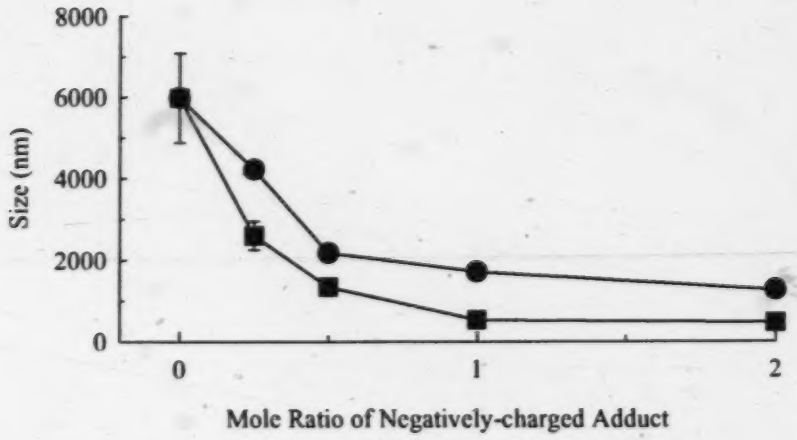


Figure 3.14. Effect of mole ratio of charged adduct on the size of DPPC:CH liposomes (7:2 m.r.) prepared by the CF method, ●, DMPG; ■, DPPG. Means ±SE, n=3.

Table 3.3. Liposome size as a function of preparation technique and polymer coating [a,b]

Coating Status	Preparation Method	Size (nm) NL[c]	PCL[d]	NCL[e]
Uncoated	CF[f]	3119 ± 406[h]	1052 ± 224[h]	2171 ± 149
	DRV[g]	n.d.[i]	3075 ± 241	1732 ± 75
CHM-coated	CF	3035 ± 281[h]	1128 ± 171[h]	n.d.
	DRV	n.d.	n.d.	2100 ± 223
CM-chitin-coated	CF	3114 ± 300[h]	4703 ± 396[h]	n.d.

a. Mean ± SE, n=3

b. Initial drug/lipid weight ratios used to prepare liposomes were 1/10 except for PCL prepared by the CF method which was 0.5/10.

c. DPPC:CH (7:2 m.r.) liposomes

d. DPPC:CH:SA (7:2:1 m.r.) liposomes

e. DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes

f. classic film method

g. dehydration-rehydration method

h. bath-sonicated (see section 2.2.3.1)

i. not determined.

3.3.4. DSC Study of liposomes

DSC studies were conducted to obtain evidences of the coating of liposomes by polymers. Representative DSC scans shown in Figure 3.15 illustrate the effects of CH and the charged adducts, SA and DMPG, on the T_m of DPPC. A T_m of 41.3 °C and a pre-transition temperature of 34.7 °C were observed for DPPC liposomes (Figure 3.15a). The T_m of DPPC increased to 43.5 °C but its enthalpy of transition ($\Delta H°$) decreased drastically to 9.1 mJ/mg from 48.7 mJ/mg with 22 mol percent CH incorporation (Figure 3.15b and Table 3.4). The presence of 12.5 mole percent SA increased the T_m of DPPC to 44.7 °C and also slightly increased the $\Delta H°$ to 52.0 mJ/mg. Incorporation of 6.7 mole percent DMPG decreased the T_m to 40.1 °C and the $\Delta H°$ to 40.4 mJ/mg.

The effects of insulin incorporation and polymer interaction on the thermal behavior of DPPC liposomes, DPPC:SA (7:1 m.r.) liposomes, and DPPC:DMPG (7:0.5 m.r.) liposomes are illustrated in Figure3.16 and the details are included in Table 3.4. The effect of insulin on T_m and $\Delta H°$ was negligible. CM-chitin increased $\Delta H°$ of DPPC:SA liposomes to 57.3 mJ/mg, but had no effect on the T_m. The thermal behavior of all other liposome systems was not affected by CM-chitin. Also, CHM had no effect on Tm, however, $\Delta H°$ was lowered (Table 3.4).

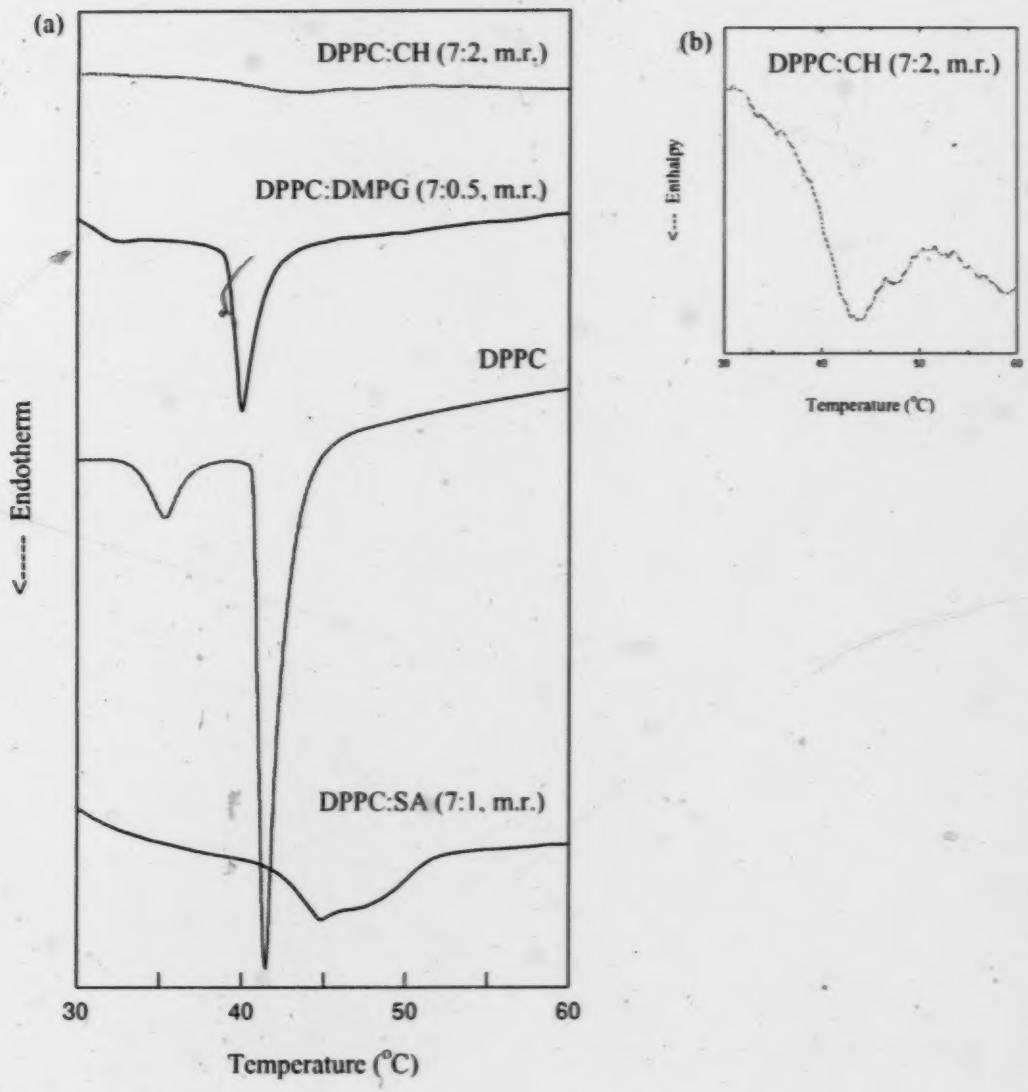


Figure 3.15. Effects of CH and charged adducts (SA or DMPG) on DSC thermograms of DPPC liposomes. Inset (b) shows enlargement of the thermogram of DPPC:CH (7:2 m.r.) liposomes from (a).

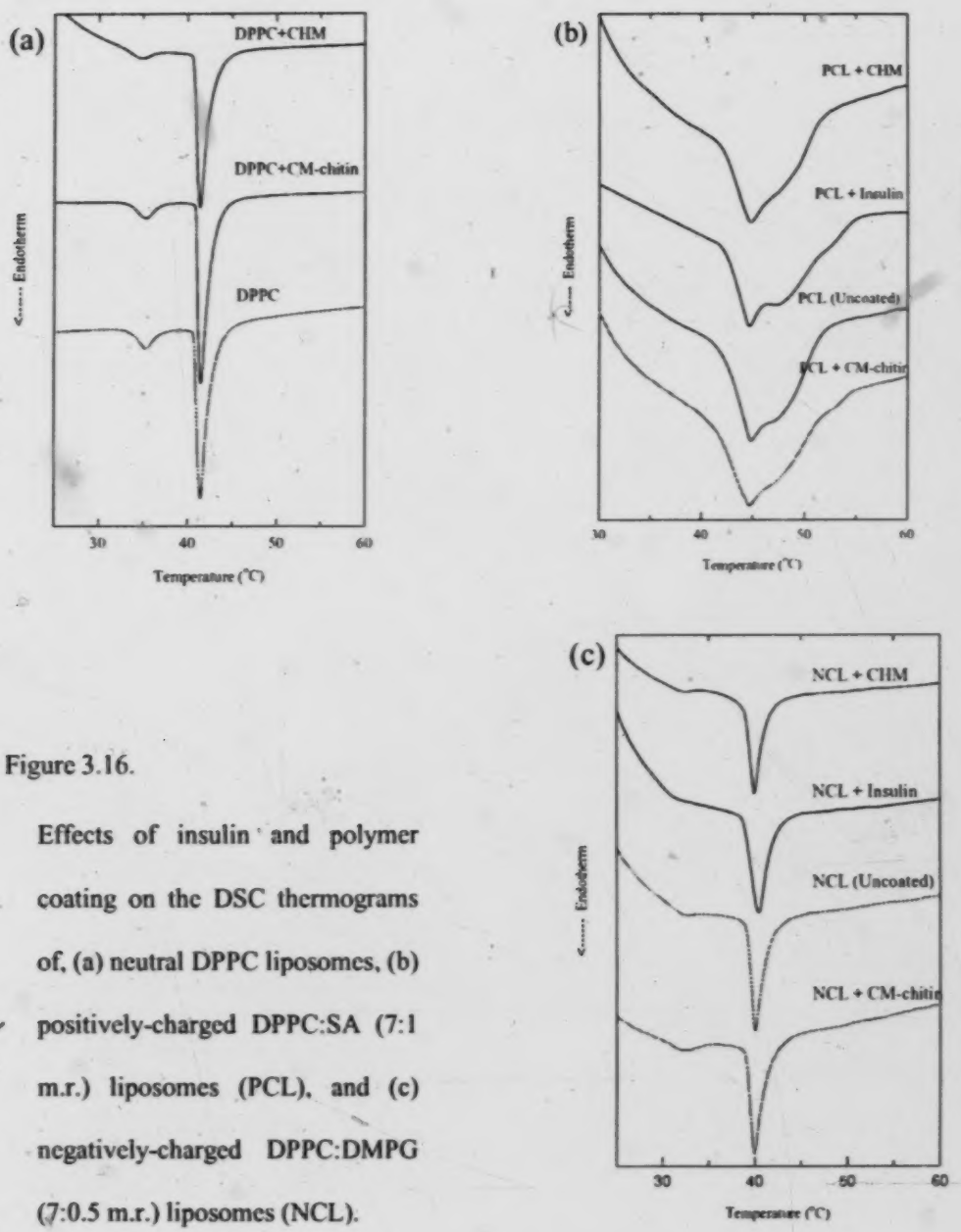


Figure 3.16.

Effects of insulin and polymer coating on the DSC thermograms of, (a) neutral DPPC liposomes, (b) positively-charged DPPC:SA (7:1 m.r.) liposomes (PCL), and (c) negatively-charged DPPC:DMPG (7:0.5 m.r.) liposomes (NCL).

Table 3.4. Thermodynamic characteristics of DPPC liposomes[a]

Liposome Composition	Pretransition Temp. (°C)	Main Transition Temp. (T_m) (°C)	Enthalpy Change ($\Delta H°$) mJ/mg
DPPC	34.7	41.3	48.7
DPPC:CH (7:2 m.r.)	-	43.5	9.1
DPPC + CM-chitin	34.7	41.3	48.2
DPPC + CHM	-	41.4	41.5
DPPC:SA (7:1 m.r.)	-	44.7	52.0
DPPC:SA (7:1 m.r.) + Insulin	-	44.7	50.4
DPPC:SA (7:1 m.r.) + CM-chitin	-	44.7	57.3
DPPC:SA (7:1 m.r.) + CHM	-	44.8	42.7
DPPC:DMPG (7:0.5 m.r.)	32.5	40.1	40.4
DPPC:DMPG (7:0.5 m.r.) + Insulin	-	40.4	41.6
DPPC:DMPG (7:0.5) + CM-chitin	-	40.0	40.5
DPPC:DMPG (7:0.5 m.r.) + CHM	32.4	40.0	36.2

[a] Liposomes contain no insilin, unless otherwise stated. Values; means of 2 or 3.

3.4. Liposome Behavior in Various Media

3.4.1. Stability of liposomes in bile salt solutions

A typical standard curve of absorbance of liposome dispersions versus lipid concentration is depicted in Figure 3.17. Relative turbidity (as a measurement of stability) of liposomes as a function of either SC concentration, or polymer coating or incubation time at pH 7.4 and various temperatures are depicted in Figures 3.18-3.20. Uncoated and CHM-coated PCL were essentially stable in a SC solutions from 0-24 mM at RT. No differences in turbidity of liposomes were observed after 15 min or 60 min incubation time (Figure 3.18). CM-chitin-coated liposomes were not as stable from 10-24 mM SC at RT. At 37 °C, however, turbidities of both uncoated and CHM-coated PCL decreased at SC concentrations higher than 10 mM, whereas turbidities of CM-chitin-coated PCL were drastically reduced at SC>2 mM and reached a plateau from 12-24 mM (Figure 3.19). Uncoated and CHM-coated NCL were also stable in SC solutions up to 24 mM. As with PCL, no differences between turbidity values after 15 min or 60 min incubation periods were observed (Figure 3.20).

3.4.2. Stability of insulin in liposomes in the presence of enzymes

The stability of insulin solution and insulin in liposomes in the presence of proteolytic enzymes is depicted in Figure 3.21. It can be seen that when insulin solutions were incubated with pepsin or α-chymotrypsin < 10 and < 12 percent insulin remained undegraded after 30 min, respectively, and < 1 and < 2 percent after 2 h. In uncoated or

CHM-coated liposomes incubated in the same media 60 - 85 percent of entrapped insulin remained undegraded over the same periods.

3.4.3. Insulin Release

The release profiles of insulin from uncoated, CM-chitin-coated, and CHM-coated liposomes are shown in Figures 3.22-3.31. Each is generally characterized by an initial rapid release phase of insulin followed by a slower rate after about 1 h. Insulin release from PCL in SGF was not affected by coating vesicles with CM-chitin (Figure 3.22). Although, CM-chitin-PCL provided a very slow release rate of insulin at pH 7.4, it was not the case in SIF (Figure 3.23). The extent of insulin released in SIF from CHM-PCL was considerably lower than that from U-PCL or CM-chitin-PCL ($P < 0.05$) (Figure 3.24.). Uncoated or polymer-coated NCL were quite stable in SGF (Figure 3.25). CHM-NCL had a slightly lower rate of insulin release than U-NCL, however, the presence or absence of pepsin seemed to have a minor effect on the insulin release rate (Figure 3.26). NCL coated with polymers had a slower rate of release in SIF than uncoated liposomes (Figure 3.27), more significant for CHM-NCL than CM-chitin-NCL ($P < 0.05$). The presence of enzymes in SIF appeared to slightly increase the leakage of insulin (Figure 3.28). The effect of liposome composition on insulin release rate is depicted in Figures 3.29 and 3.30. NCL consisting of DSPC were more stable than DPPC liposomes in both environments of SGF and SIF without enzymes. In all cases, CHM-NCL provided the least leakage rate of insulin compared to U-NCL or CM-chitin-NCL.

Figure 3.31 demonstrates the effect of liposome treatment on the assessment of the release rate of the drug in SGF. Overestimation of the release rate of insulin measured by HPLC was the result of frequent washing stages required to deactivate residual enzymes on the surfaces of liposomes, whereas where such washings were not required, i.e. in the case of insulin release in SGF in the absence of pepsin or in the case of direct measurement of ^{125}I-insulin in SGF by γ-counting, no significant differences in the %drug released were observed (Figure 3.26).

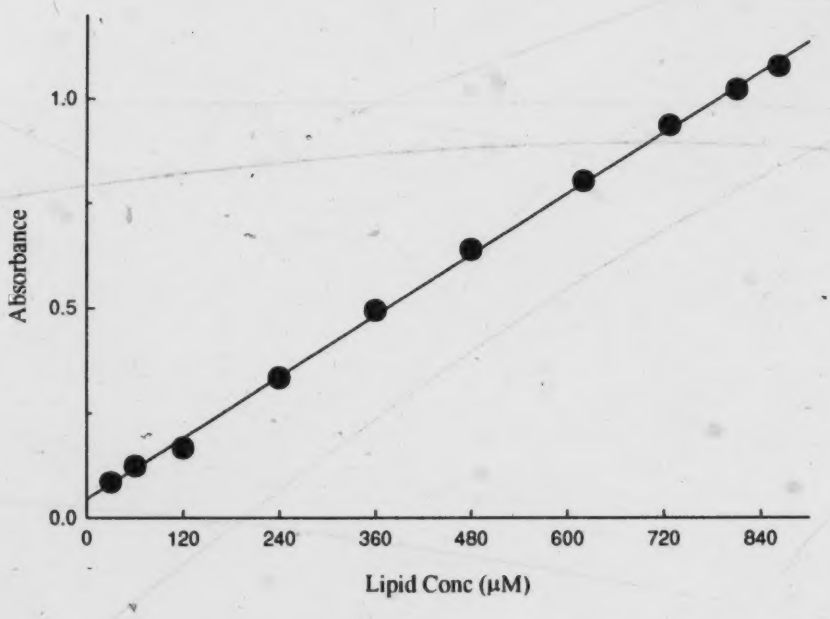


Figure 3.17. An absorbance standard curve of DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes as a function of lipid concentration at 400 nm and RT.

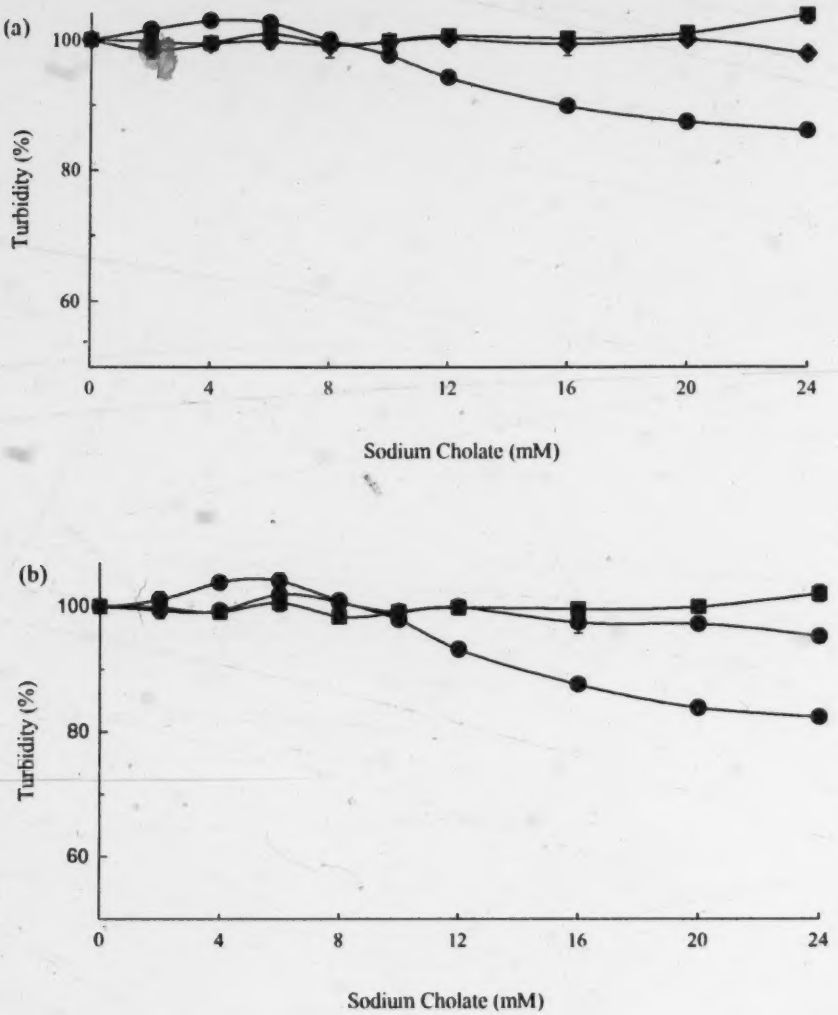


Figure 3.18. Relative stability of DPPC:CH:SA (7:2:1 m.r.) liposomes as a function of SC concentration at pH 7.4 and RT; (a) after 15 min, and (b) after 60 min incubation. ■, CHM-coated; ●, CM-chitin-coated; and ◆, uncoated liposomes. Mean ± SE, n=3.

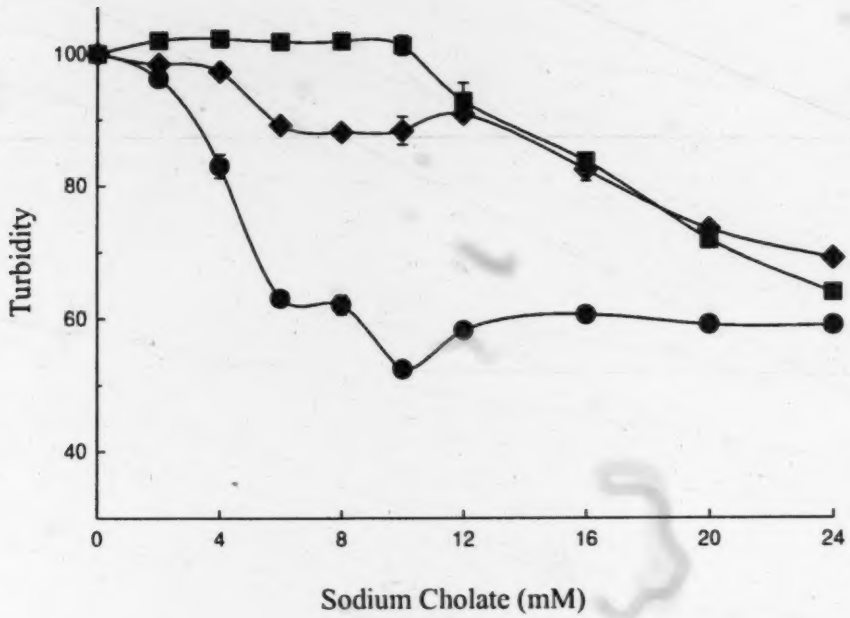


Figure 3.19. Relative stability of DPPC:CH:SA (7:2:1 m.r.) liposomes as a function of SC concentration after 15 min incubation at pH 7.4 and 37°C. ■, CHM-coated; ●, CM-chitin-coated; and ◆, uncoated liposomes. Means ± SE, n=3.

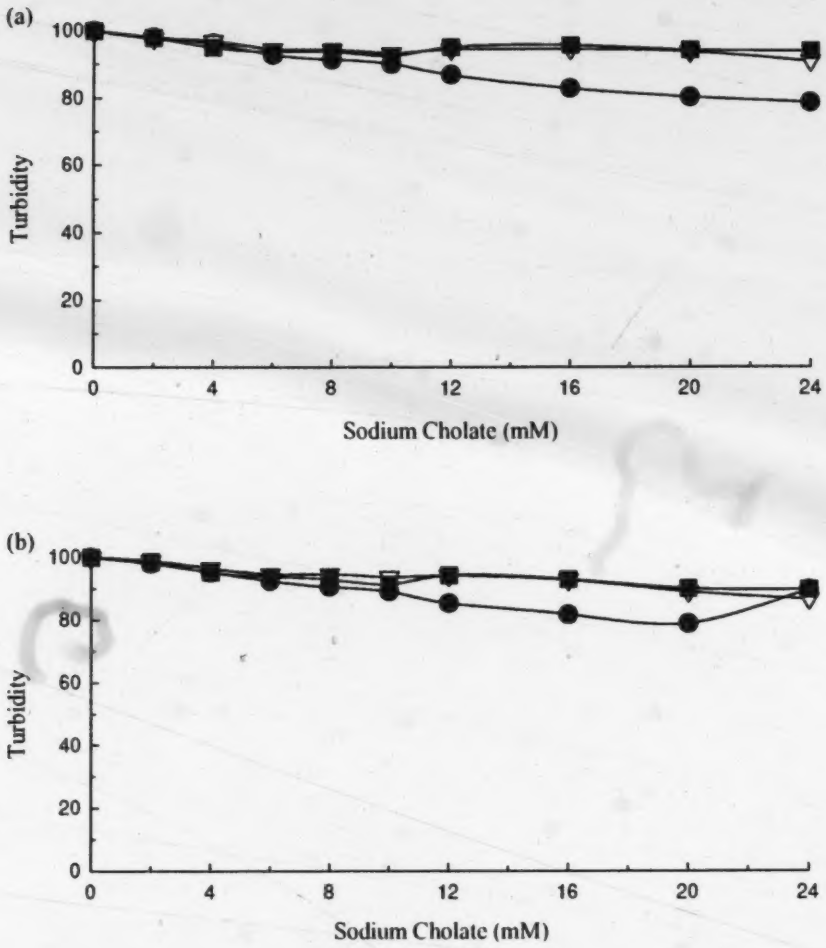


Figure 3.20. Relative stability of DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes as a function of SC concentration at pH 7.4 and RT. (a) after 15 min, and (b) after 60 min incubation. ■, CHM-coated; ●, empty liposomes; and ▽, uncoated liposomes, Means ± SE, n=3.

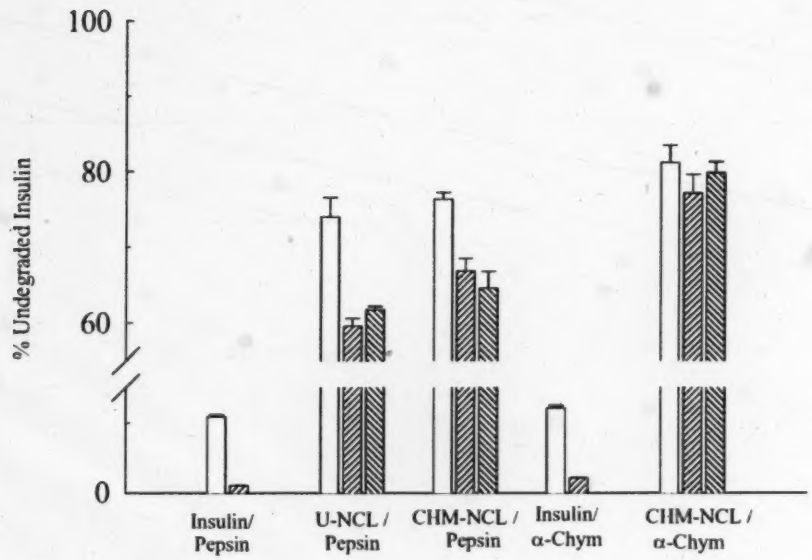


Figure 3.21. Stability of insulin solution or insulin encapsulated in uncoated- or CHM-coated- NCL (DPPC:CH:DMPG, 7:2:0.5 m.r.) in the presence of pepsin or α-chymotrypsin at 37 °C after 0.5 h, ▭; 2 h, ▨; or 4 h, ▩ incubation period. Means ± SE, n=3.

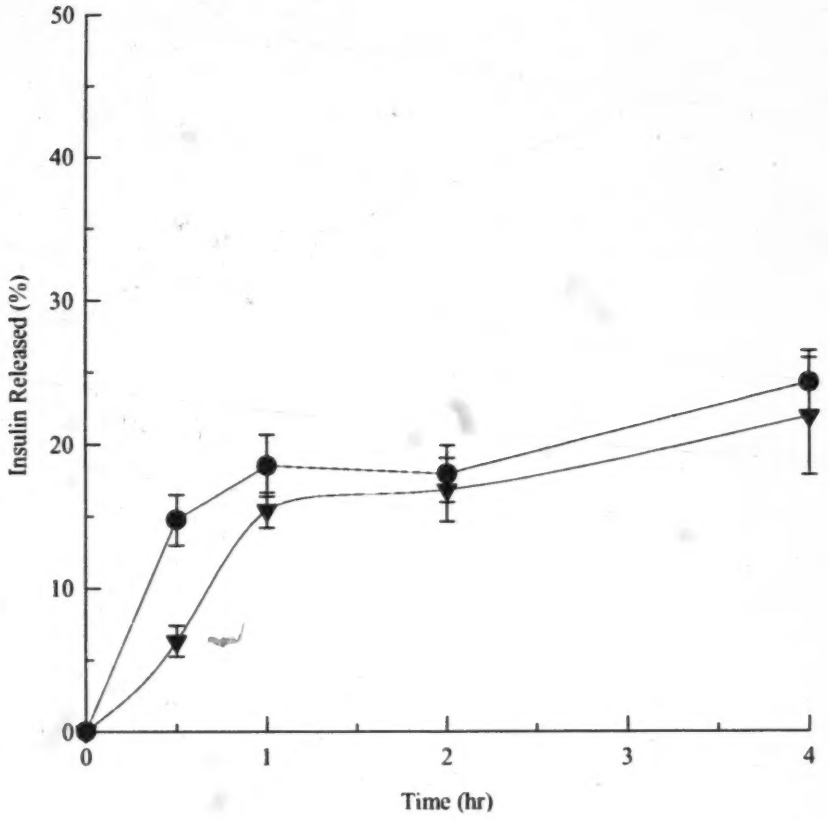


Figure 3.22. Insulin release from DPPC:CH:SA (7:2:1 m.r.) liposomes in SGF at 37 °C. ●, uncoated and ▼, CM-chitin-coated.

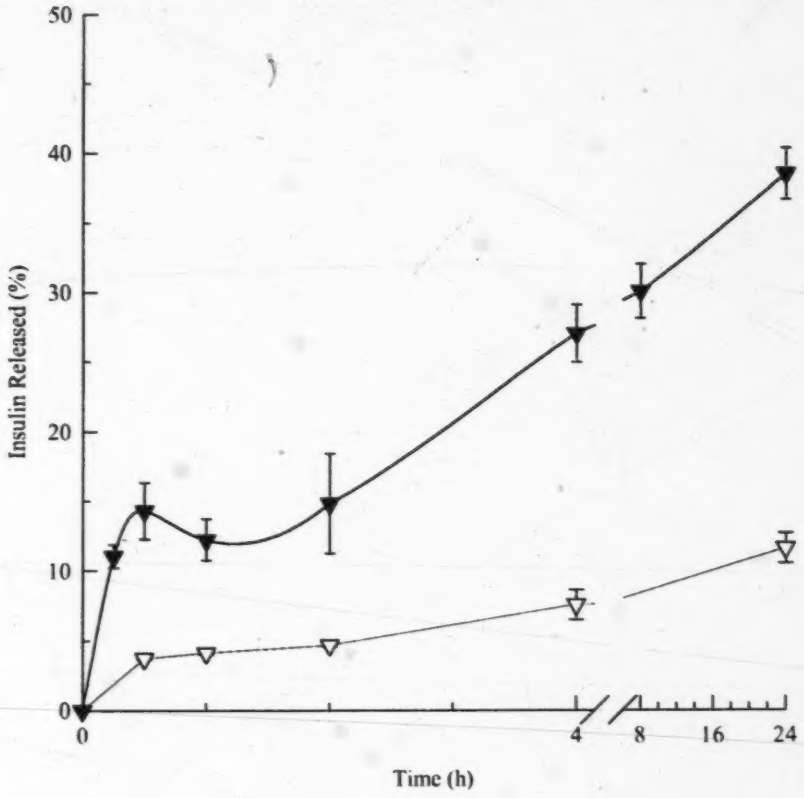


Figure 3.23. Comparison of insulin release from CM-chitin-PCL in PBS (▽) and in SIF (▼) at 37 °C. Means ± SE, n=3.

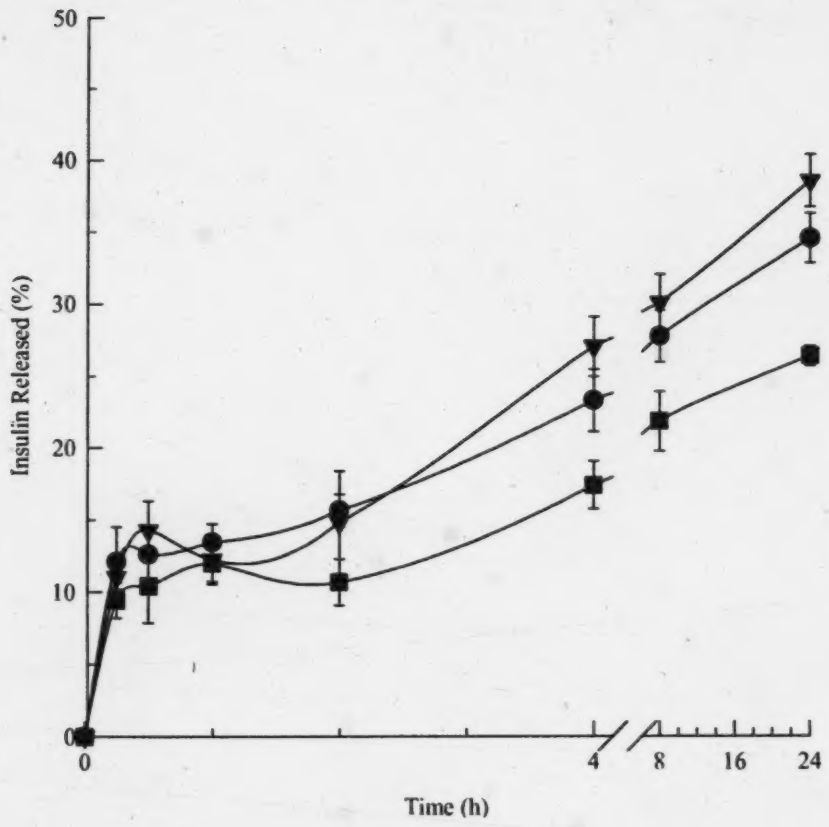


Figure 3.24. ^{125}I-insulin release from DPPC:CH:SA (7:2:1 m.r.) liposomes in SIF at 37 °C. ●, uncoated, ■, CHM-coated, and ▼, CM-chitin-coated.

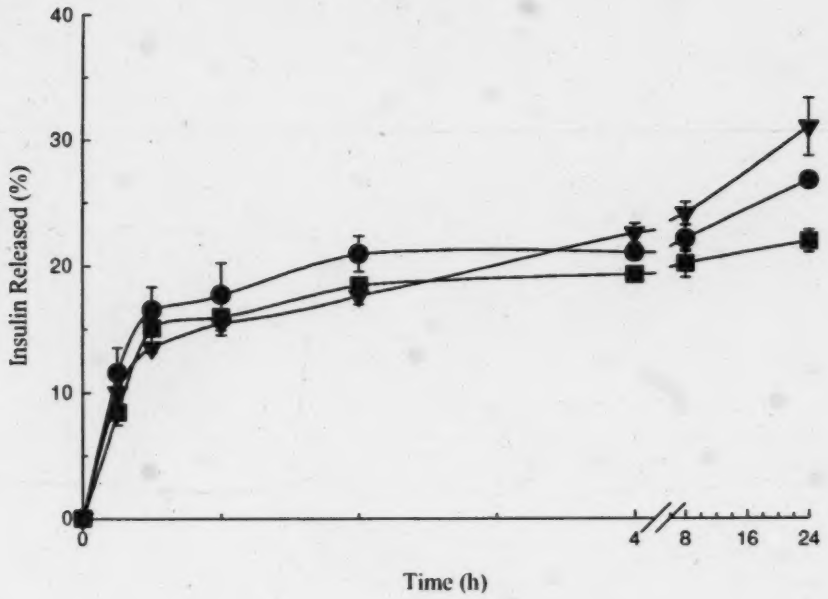


Figure 3.25. ^{125}I-insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SGF at 37 °C. ●, uncoated; ■, CHM-coated; and ▼, CM-chitin-coated.

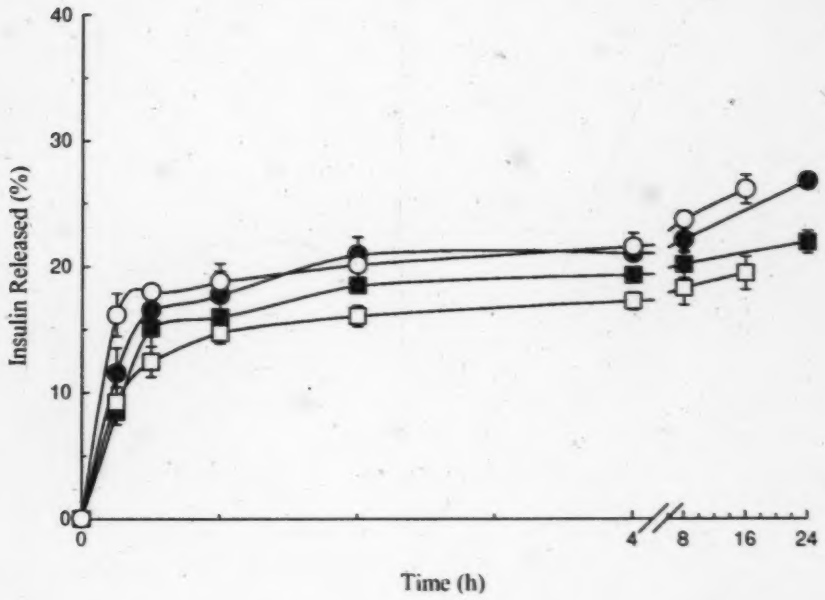


Figure 3.26. Comparison of insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SGF (closed symbols) or SGF without enzymes (open symbols) at 37 °C. Uncoated (circles); CHM-coated (squares).

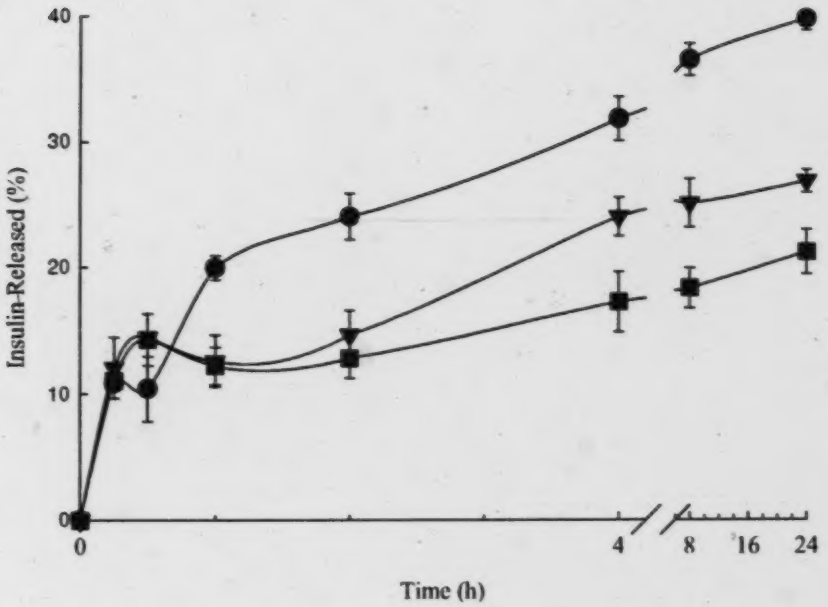


Figure 3.27. ^{125}I-insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SIF at 37 °C. ●, uncoated; ■, CHM-coated; ▼, CM-chitin-coated.

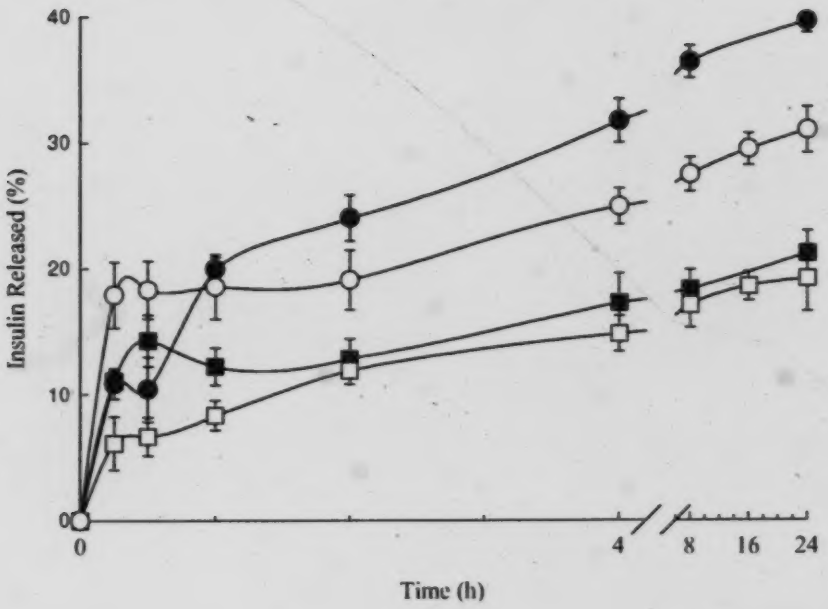


Figure 3.28. Comparison of insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SIF (closed symbols) or SIF without enzymes (open symbols) at 37 °C. Circles, uncoated; and squares, CHM-coated.

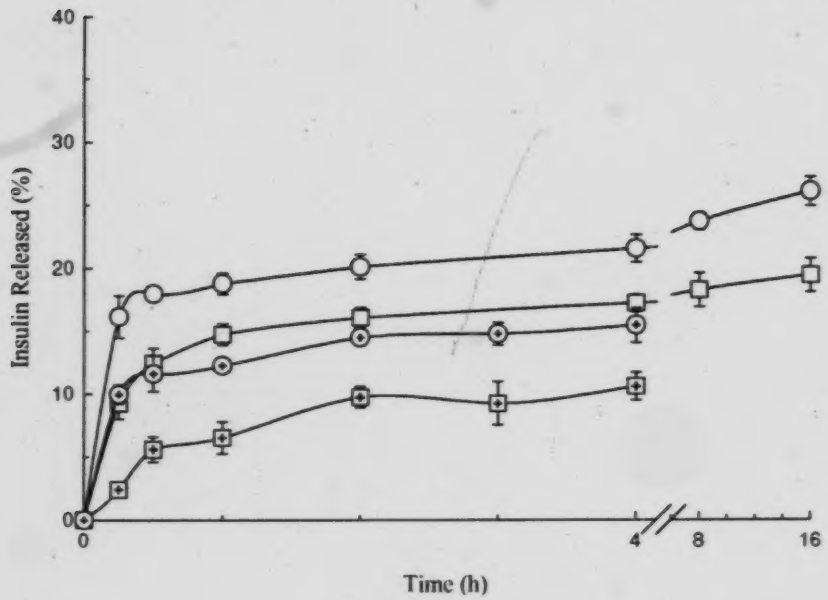


Figure 3.29. Release of insulin in SGF without enzymes at 37 °C as a function of liposome composition and polymer coating. Open symbols, DPPC:CH:DMPG (7:2:0.5 m.r.); open symbols with plus signs, DSPC:CH:DMPG (7:2:0.5 m.r.). Circles, uncoated, and squares, CHM-coated.

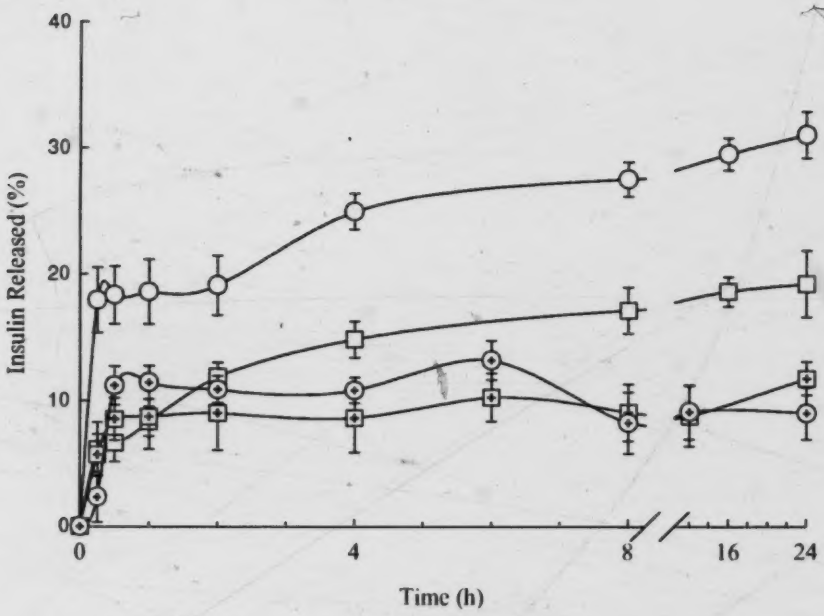


Figure 3.30. Release of insulin in SIF without enzymes at 37 °C as a function of liposome composition and polymer coating. Open symbols, DPPC:CH:DMPG (7:2:0.5 m.r.); open symbols with plus signs, DSPC:CH:DMPG (7:2:0.5 m.r.). Circles, uncoated; squares, CHM-coated.

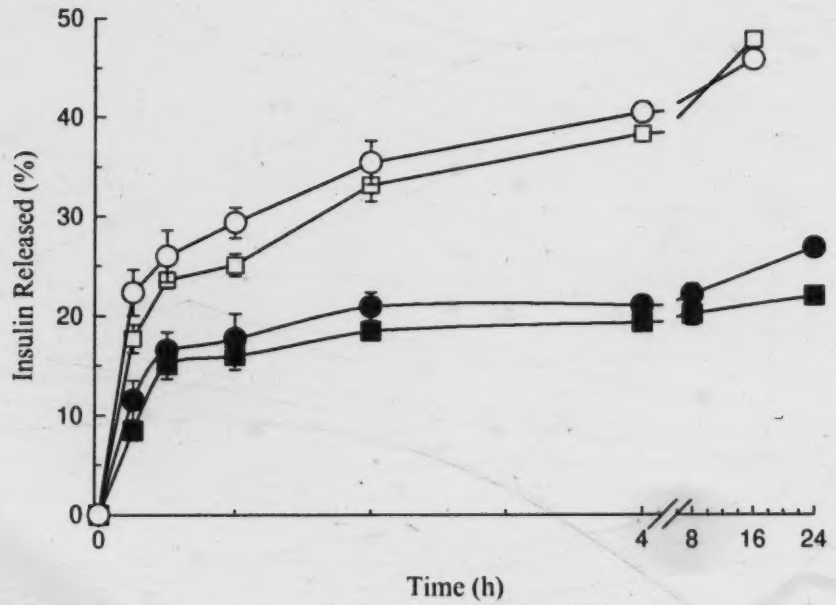


Figure 3.31. Effect of the method of treatment of liposomal insulin on the assessment of drug release. Comparison of insulin release from DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes in SGF at 37 °C. Closed symbols, direct determination of release of ^{125}I-insulin by γ-counting; open symbols, determination of insulin in liposomal pellet after deactivation of pepsin in the medium. Circles, uncoated; squares, CHM-coated.

NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

UMI

NCL significantly reduced blood glucose levels over the 4 h to 12 h period compared to the control (Figure 3.35). In contrast, U-NCL showed a mild effect (~ 70% of baseline) only after 12 h which was not significantly different from the control (Figure 3.35). When the same dose of insulin administered via positively-charged liposomes, lower reductions of blood glucose levels were observed for CHM-PCL compared to U-PCL (Figure 3.36). In order to compare the effect of liposome charge on absorption of insulin, the curves in Figures 3.35 and 3.36 were rearranged to reflect this effect (Figure 3.37). Hypoglycemic effect of U-PCL was higher than U-NCL (Figure 3.37a), while CHM-NCL demonstrated a stronger effect than CHM-PCL (3.37b).

Figure 3.38 shows blood glucose profiles after various doses of oral CHM-NCL. Area above glucose concentration curves, $AAC_{0\text{-}12h}$, calculated from these curves were found to have a high correlation ($r^2 > 0.95$) with the dose of insulin ranging 35-400 U/kg (Figure 3.39). $AACs_{0\text{-}12h}$ of all formulations depicted in Figure 3.40 show that insulin even as a solution slightly decreased the blood glucose level compared to PBS or empty liposomes and that U-NCL was the least effective formulation among insulin liposome formulations. The overall effect of CHM-PCL 280 (i.e. equivalent to 280 U/kg insulin) was comparable to that of a much smaller dose of 35 U/kg of CHM-NCL 35. U-PCL 280, though more effective than CHM-PCL 280, was considerably less effective than CHM-NCL 280 and its effect was comparable with CHM-NCL 70. The effect of CHM-NCL 400 ($AAC_{0\text{-}12h}$) was equivalent to subcutaneous injection of 2 U/kg insulin (Figure 3.40). While the effects of the latter was already diminished at 12 h after injection (Figure 3.34), the former seemed to be continuing its hypoglycemic activity (Figure 3.38).

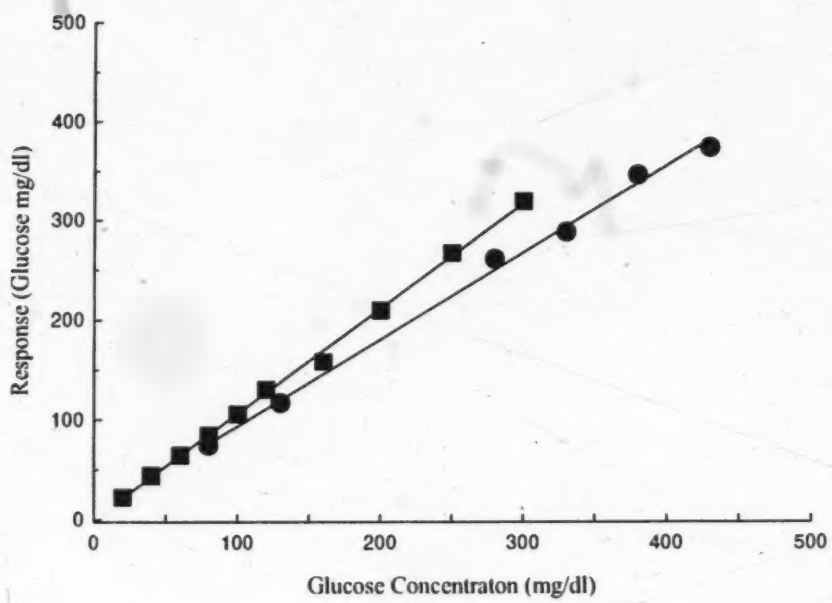


Figure 3.32. The calibration curve of glucose measured by a Beckman® Astra-8® analyzer (■) or a Medisense® blood glucose sensor electrode apparatus (●). Units of mmol/l obtained by the Medisense® apparatus were converted to mg/dl by multiplying by 18.02.

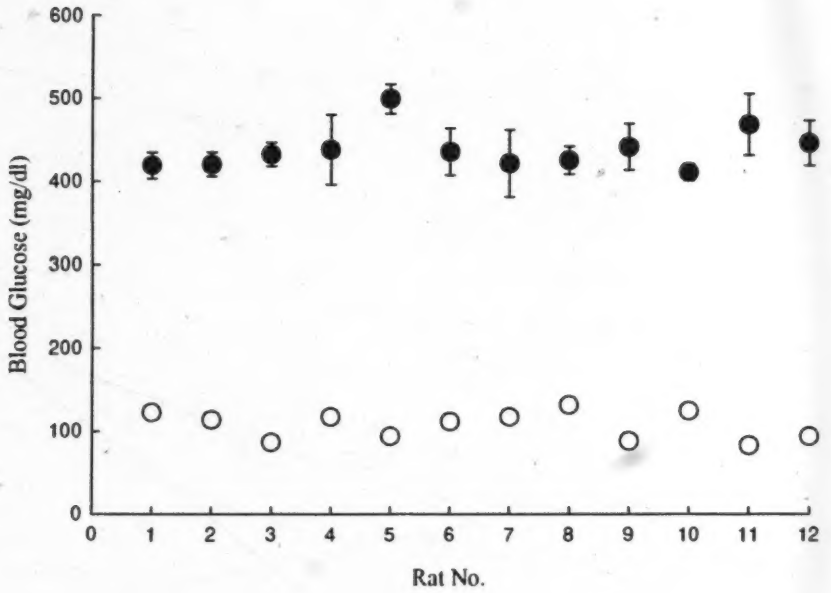


Figure 3.33. Typical blood glucose levels of rats with (●) or without diabetes (○). Diabetes was induced by i.v. injection of 55 mg/kg streptozocin (STZ) via the pineal vein and blood glucose sampling occurred on days 7, 8, and 9 after STZ injection. Means ± SE, n = 3.

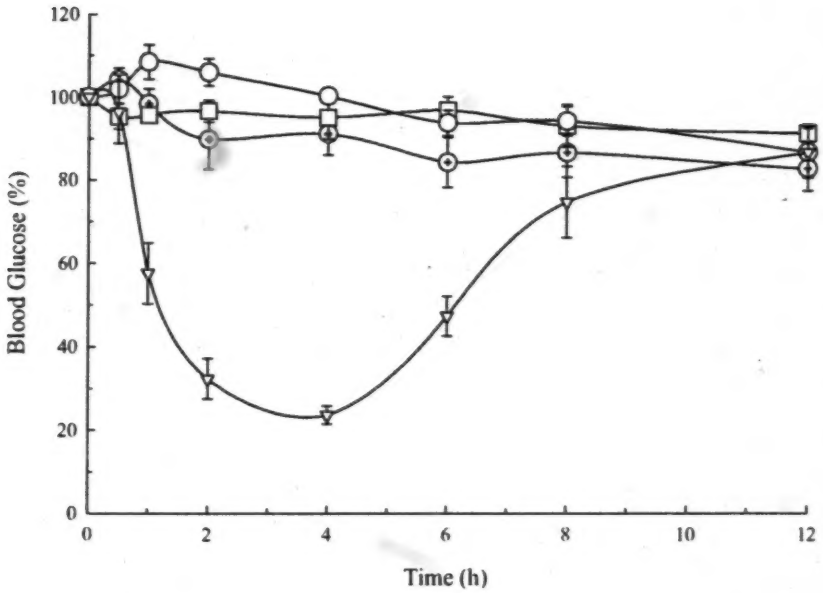


Figure 3.34 Blood glucose levels (%) in fasted diabetic rats (control groups) following intragastric administration of (⊙), 400 U/kg of insulin solution, (□), pH 7.4 PBS solution, (○), empty liposomes, or (▽) s.c. injection of 2 U/kg insulin. Means ± SE, n = 6 or 8.

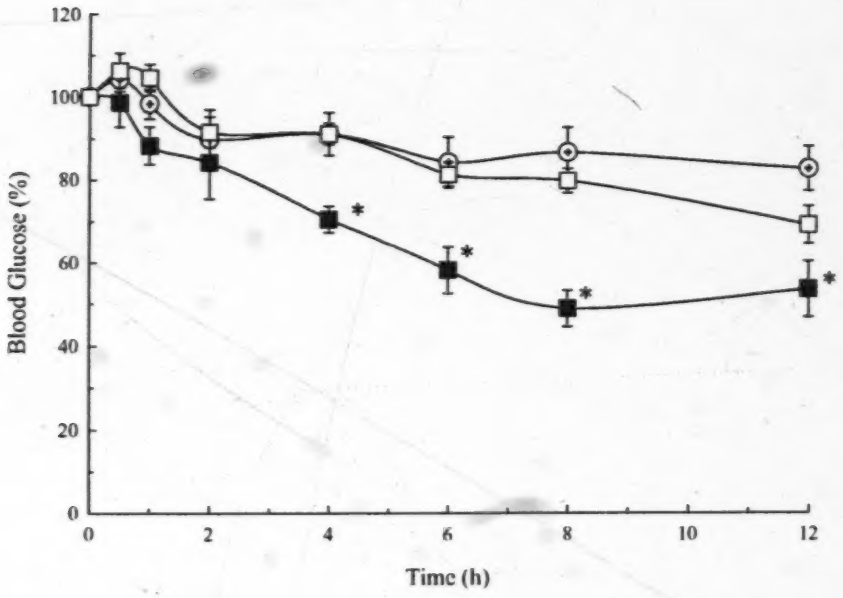


Figure 3.35. Comparison of the hypoglycemic effects of insulin-containing uncoated or CHM-coated negatively-charged liposomes (CHM-NCL) following intragastric administration of a formulation to fasted diabetic rats. (□), 280 U/kg insulin in U-NCL; (■), 280 U/kg insulin in CHM-NCL; (⊙), 400 U/kg insulin solution (as control group). Means ± SE, n = 6 - 8; *, $P < 0.05$.

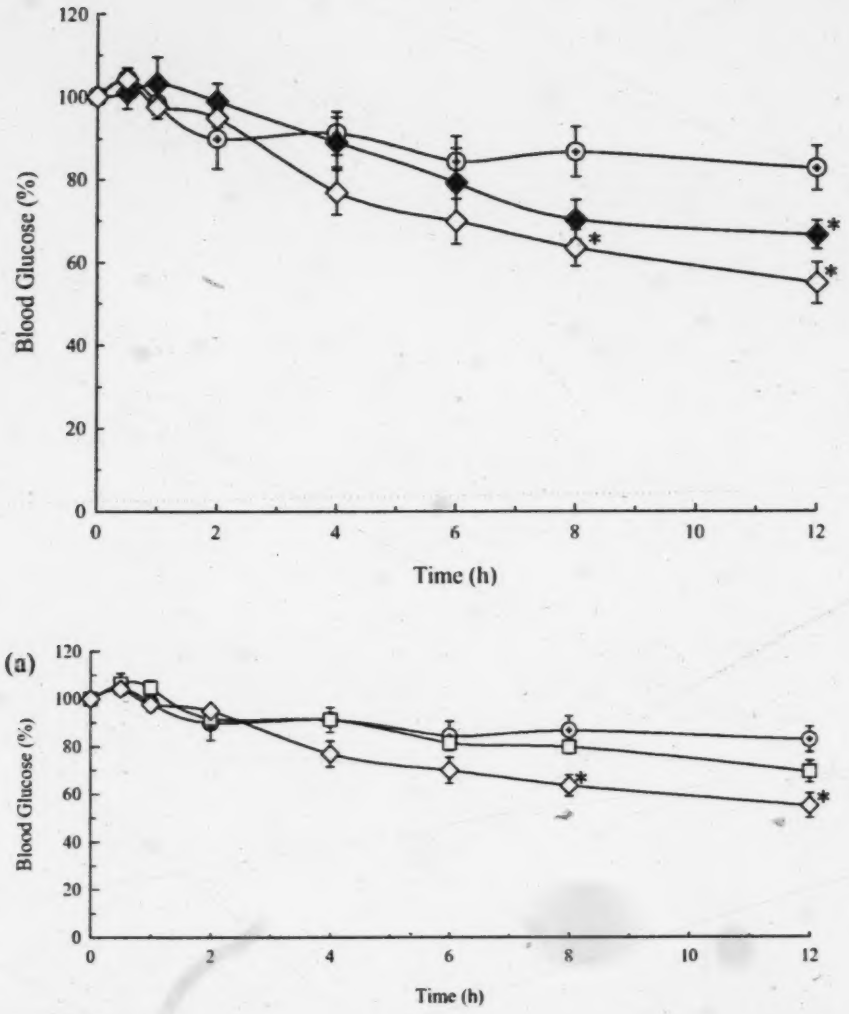


Figure 3.36. Comparison of the hypoglycemic effects of insulin containing uncoated or CHM-PCL following intragastric administration to fasted diabetic rats. (◇), 280 U/kg insulin in U-PCL; (◆), 280 U/kg insulin in CHM-PCL; (⊙), 400 U/kg insulin solution (as control). Means ± SE, n = 6 - 8; *, $P < 0.05$

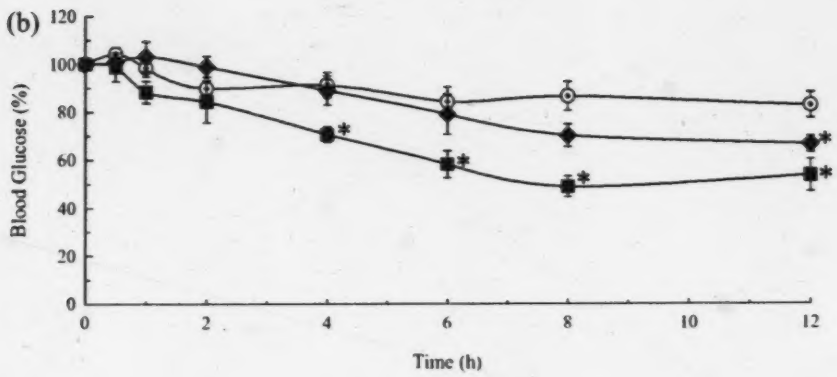


Figure 3.37. Comparison of the hypoglycemic effects of insulin liposomes following intragastric administration to fasted diabetic rats as a function of liposome charge. (a) uncoated, and (b) CHM-coated. (◇), 280 U/kg insulin, U-PCL, (◆), 280 U/kg insulin, CHM-PCL, (□), 280 U/kg insulin, U-NCL, (■), 280 U/kg CHM-NCL, and (⊙), 400 U/kg insulin, solution (as control). Curves were extracted from figures 3.35 and 3.36 and rearranged. Mean ± SE, n = 6 - 8, *, $P < 0.05$

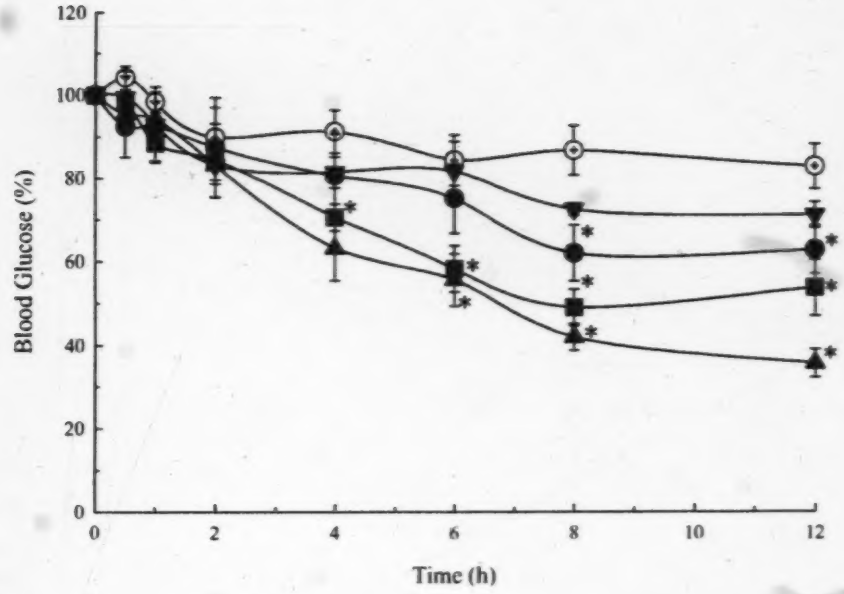


Figure 3.38. Blood glucose levels (%) following intragastric administration of various doses of insulin as CHM-NCL to fasted diabetic rats. (▼), 35 U/kg; (●) 70 U/kg; (■), 280 U/kg; (▲), 400 U/kg; (⊙), 400 U/kg of insulin solution (as control). Means ± SE, n = 6 - 8, *, $P < 0.05$

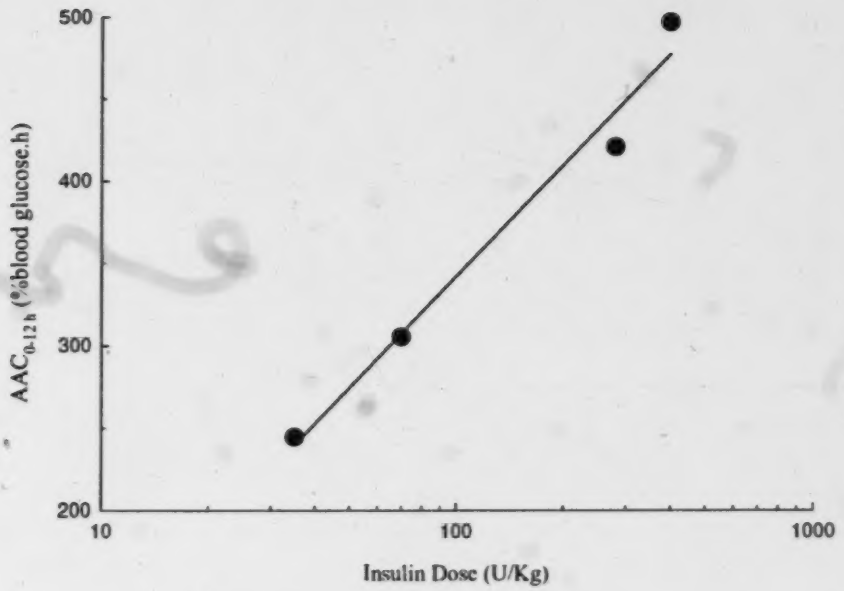


Figure 3.39. Dose-effect relationship of insulin encapsulated in CHM-NCL formulations intragastrically administered to fasted diabetic rats ($r^2 > 0.95$).

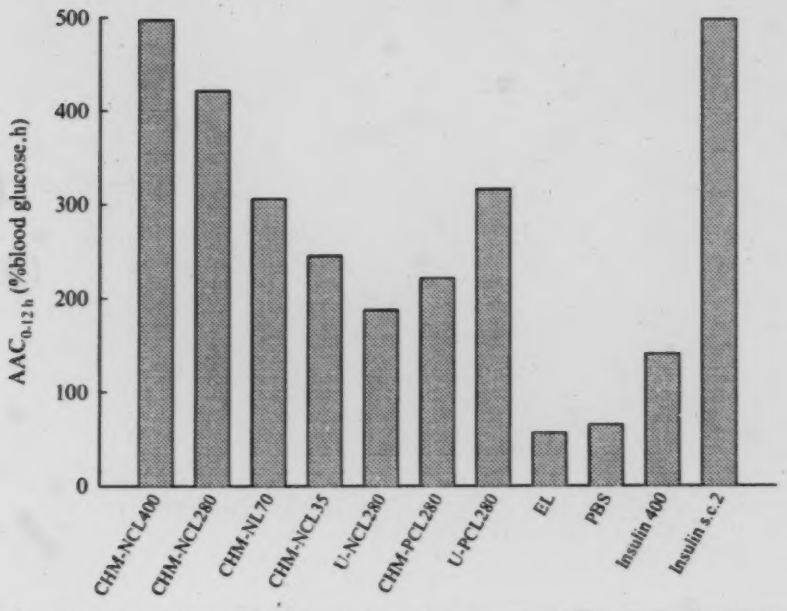


Figure 3.40. Comparison of the area above the plasma glucose concentration-time curves (%blood glucose.h), $AAC_{0\text{-}12h}$, of intragastrically-administered formulations in fasted diabetic rats. The base line was the mean of blood glucose concentrations prior to administration of formulations. CHM-, cholesteryl-mannan; U, uncoated; NCL, negatively-charged liposomes consisted of DSPC:CH:DMPG (7:2:0.5 m.r.) prepared by DRV method; PCL, positively-charged liposomes consisted of DSPC:CH:SA (7:2:1 m.r.) prepared by DRV method; PBS, pH 7.4 phosphate buffer saline solution; Insulin, insulin solution; Insulin s.c., subcutaneously injected insulin; and EL, empty liposomes. The number after each abbreviation represents the insulin dose administered.

4. DISCUSSION

The results of studies with polymer-coated liposomes are indicative of the ability of CHM to provide greater coating and decreased release of insulin under conditions of SGF and SIF compared to uncoated or CM-chitin-coated liposomes. Furthermore, negatively-charged liposomes were superior to positively-charged liposomes when coated with polymers. The levels of insulin which were retained in CHM-coated negatively-charged liposomes after 1 h incubation in SGF and 6 h in SIF were 85 and 80 percent, respectively. It can be estimated that if liposomes were transported from SGF to SIF, then after 6h approximately 70 percent of the initial amount of insulin would be available in the intestine for absorption. The in vivo results also indicated that CHM-coated negatively-charged liposomes promoted oral absorption of insulin more efficiently than other formulations.

4.1. Polymer Derivatization

The major modification to the method of cholesterol derivatization of polysaccharides, previously reported by Sunamoto *et al.* [164,165], was the first step, i. e. carboxymethylation (Figure 4.1, comparison of steps a-c with d). Alcohols in aqueous media usually have an equilibrium reaction with sodium hydroxide because of their higher pKa:

But sodium hydride in an aprotic environment reacts more vigorously with alcohols producing hydrogen gas, which upon removal, shifts the reaction to the right direction:

Accordingly, the primary hydroxyl group of the polysaccharide molecule **(1)** which was a weak alcohol formed alkoxide **(2)** more easily with NaH in an aprotic environment (dry DMSO) than with NaOH in water (compare Figure 4.1a and 4.1d).

Reacting **(2)** with bromomethyl acetate in dry DMSO, rather than with sodium chloroacetate in water (compare Figure 4.1b with 4.1d), resulted in production of carboxymethyl-mannan methyl ester **(3)** which was easily transformed to carboxymethyl-mannan **(4)** upon hydrolysis in alkaline pH (Figure 4.1c). The bromine is a better leaving group than chlorine, therefore, a better yield was expected. In fact, improved derivitization of carboxymethyl on polysaccharides using this method resulted in greater reproducibility in the derivitazation of polysaccharide with cholesterol.

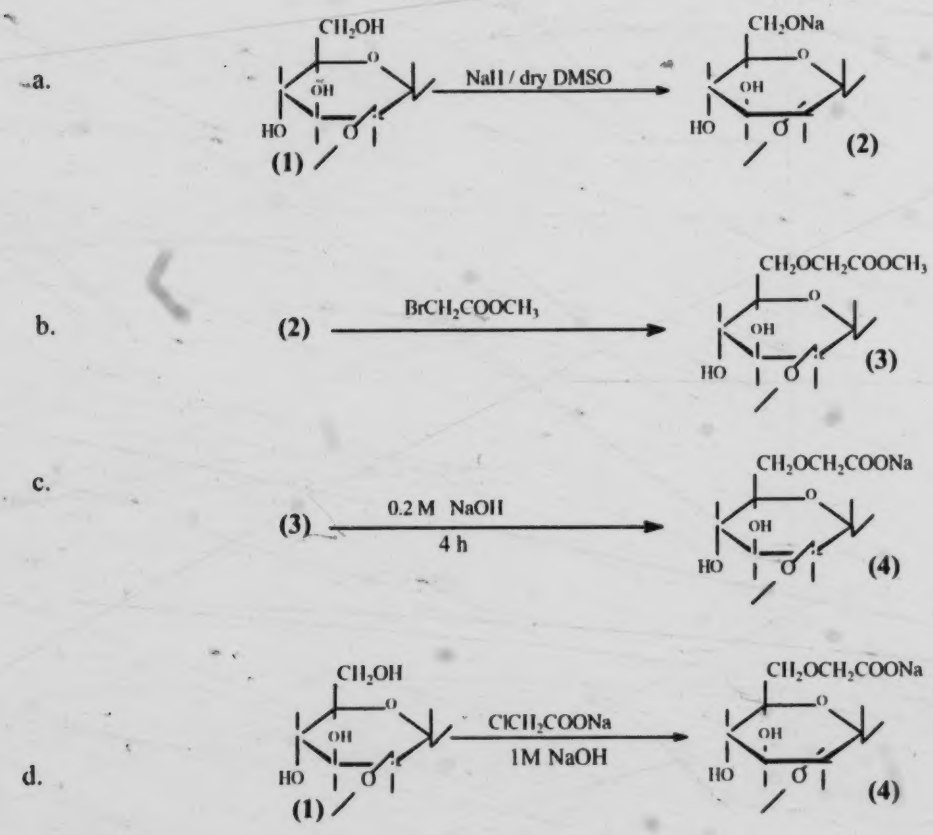


Figure 4.1. The modified carboxymethylation steps of polysaccharides (e.g. mannan) derivatization (a-c) compared to single derivatization step of Inman [205], step d.

Further derivatization of carboxymethyl polysaccharide (compound **(4)** from figure 4.1) with ethylendiamine and subsequent cholesterol (Figure 4.2) was carried out according to earlier reports [164,165,205].

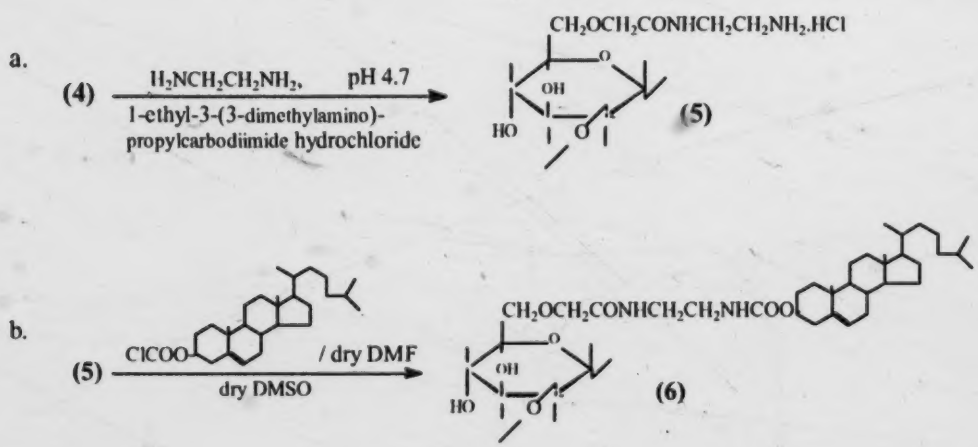


Figure 4.2. Ethylenediamine (a) and cholesterol (b) derivatization of carboxymethyl polysaccharides (e.g. mannan) as described in references [164,165,205], yielding the final cholesterol aminoethylcarbomethyl (AECM) polysaccharide, e.g. CHM.

The second step of amide formation with ethylenediamine (4.2a) was carried out in a large excess of diamine in order to minimize cross-linking through formation at both amino groups of any given diamine molecules [205]. Since reactions involving

carbodiimide were carried out at pH 4.7, the concentration of reactive, uncharged amino group is greatly augmented because of the opposite charge of the opposing protonated amino group. After reaction of diamine molecule with an activated carboxyl, the bound amino group would compete unfavorably with an unreacted diamine molecule since a much smaller fraction would be in the reactive, uncharged form. In addition, unreacted species are always present in much higher concentration. Therefore, cross-linking should occur only rarely [205].

4.2. Analytical

Siliconizing the glass surfaces is a common way to prevent loss of drugs due to the adsorption. Coating glasswares with Surfasil® and DMDCS prevented loss of insulin particularly evident at low concentrations (Figure 3.4). However, siliconized flasks prevented the formation of thin lipid films, a requirement for successful preparation of liposomes. Therefore, experiments were carried out without using siliconized glassware.

The loss of insulin was also found to be reduced in the presence of CM-chitin, used to coat liposomes. Figure 4.3 shows that the HPLC response was considerably less than when insulin samples lacked the polymer. This result may be attributed to either competition of polymer with insulin for adsorption sites on the glass surface, or to a stabilizing effect of polymer in reducing peptide aggregation [206]. The mechanisms by which excipients stabilize proteins against aggregation or decomposition reactions are not well understood. However, stabilizing effects of carbohydrates have been explained based on their influence on the structure of water which determines the strength of hydrophobic

interactions. Arakawa *et al.* [207] hypothesized that proteins in the solid state were stabilized by sugars which act as an active water substitute to satisfy hydrogen bonding. Hydrophobic interactions are stronger in a sugar solution than in pure water [208]. Also, since sugars have lower dielectric constants than water, the electrostatic interactions are likely to be stronger. The stability of α-chymotrypsin was drastically improved after complexation with cholesterol-derivatized pullulan (CHP) [209]. Multipoint interactions between protein and self-aggregates of the hydrophobized polysaccharide, CHP, were believed to be the key factors in stabilization. Similarly, CHM or CM-chitin protection of insulin against adsorption and aggregation may be explained by interaction of the hydrophobic side chains of aromatic amino acid groups of the peptide with hydrophobic regions of the polymers. A similar mechanism has been proposed for the stabilizing effects of peptides by hydroxypropyl-ß-cyclodextrins [210,211].

The larger peak area of insulin (resolved from HPLC column) from insulin solution containing CTAB than from those containing polymer is evidence of better performance of surfactant than polymer in preventing insulin adsorption. A slightly smaller peak area obtained from insulin solution containing CTAB and polymer than that of insulin solution containing CTAB alone may be attributed to a lowering of free CTAB concentration as a result of its interaction with the oppositely-charged polymer at pH 7.4.

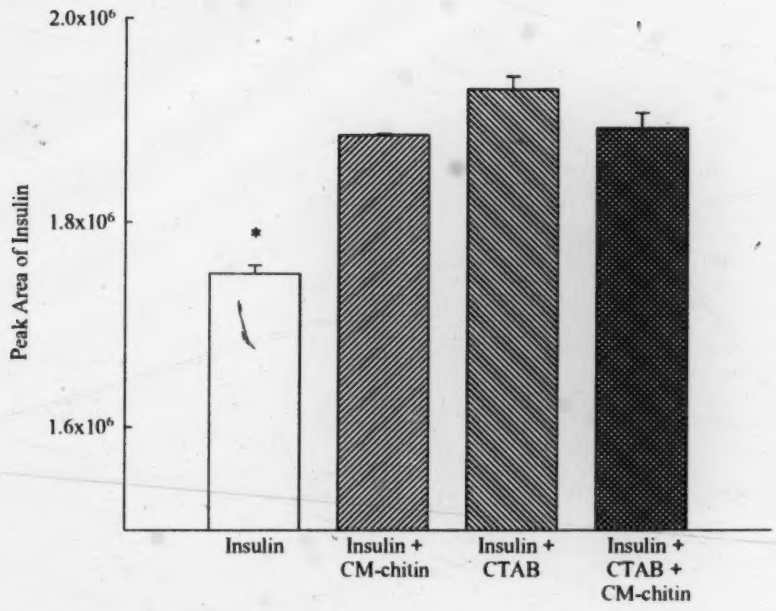


Figure 4.3. Effects of CM-chitin and CTAB on the peak area of insulin resolved from HPLC. Means ± SE (n=3), *, $P < 0.05$.

4.3. Liposome Characterization

4.3.1. Coating Efficiency (CE)

In using CTAB to disrupt liposomes in the determination of insulin content, polymer-coated liposome systems were cloudy, rather than transparent, and, indeed became the basis for measuring CE of liposomes. The resulting turbidities were due to the interaction of cationic CTAB with polymers, which were somewhat more prominent for negatively-charged CM-chitin than for neutral CHM, as is evident from their standard curves (Figure 3.5). A problem associated with turbidity methods is the possibility of precipitation of

suspended particles during the course of the experiment, giving rise to potential unreliability of absorbance measurements. This problem was overcome using CTAB because of stability of cloudy suspensions over a long period of time (Figure 3.5). The amphiphilic characteristic of CTAB seemed to be aiding particles to stay suspended, resulting in more reliable turbidometric measurements.

The degree of liposome coating depends on the nature of the interaction of polymers with liposome surfaces. It has been revealed that polysaccharides such as dextran and pullulan once adsorbed at liposome surfaces easily desorb upon dilution [212,213]. This problem has been overcome by hydrophobizing polymers by chemically conjugating cholesterol or fatty acids that tightly anchors the polymer to the bilayer [125]. A higher CE of 10% by equilibration of liposomes with 1% CHM was obtained compared to 3% CE from 1% CM-chitin (Figure 3.6). A concentration-dependent CE of liposomes by CHM (Figure 3.6b.) is comparable to that of some hydrophobized polysaccharides reported previously [214].

4.3.2. Encapsulation Efficiency (EE)

Disruption of liposomes is required for determination of the EE of entrapped drug. Membranes and membrane proteins have been solubilized by a wide variety of detergents [215,216]. Many attempts have been carried out to rationalize the specific use of detergents [217-224], however, selection of an appropriate detergent is, still in many cases, based on trial and error. Meanwhile, the astonishing recommendation of Racker [225] still holds: "Try them all!". Triton X-100 and SDS are widely used to solubilize

bilayers, but were not suitable in this instance. Triton X-100 produced many interfering peaks in the HPLC chromatograms of insulin, whereas SDS caused the insulin HPLC peak to broaden and be unresolved from its desamido derivative, a possible degradation product. In contrast to these detergents, CTAB caused a sharp and well-resolved insulin peak with no interference. Also, CTAB was a better solubilizing agent. This was evident from the amount of insulin detected after solubization of liposomes by various detergents (Figure 3.7).

The mechanisms of insulin entrapment were different for PCL and NCL. Insulin, having a pI of 5.6, is negatively-charged at pH 7.4, whereas stearylamine (SA) is positively charged. Therefore, entrapment of insulin in PCL is mainly due to the interactions of oppositely-charged molecules. That is evident in Figure 3.7. To evaluate the extent of association of insulin with the outer surface of liposomes, empty liposomes which were incubated with insulin solution produced only 8.3% association (Table 4.1), indicating that most of the drug was encapsulated in inner lamellae of liposomes. In comparison, negatively-charged DMPG liposomes also increased the EE of insulin compared to neutral liposomes. In this case, the main factor was increased volumes of aqueous compartments of the liposomes due to the electrostatic repulsion between adjacent bilayers. On the other hand, relatively high EE of insulin in NCL, both carrying a negative charge at pH 7.4, may suggest that hydrophobic interactions brought about by the exposure of hydrophobic residues at the protein surface are able to overcome the unfavorable electrostatic repulsion, as was suggested for interaction of partially-folded conformations of α-lactalbumin with negatively-charged membranes [226]. Lower EEs of

insulin at DMPG concentrations > 0.5 m.r. (Figure 3.8) may be attributed to less dense packing of the bilayers (decreased cooperativity of the molecules) with the result of increased leakage of insulin during preparation. Generally speaking, the CF method does not produce liposomes with high EE, however, insulin EE in NCL prepared by the CF method reached 22%, when insulin was incorporated from a small volume of concentrated solution (Figure 3.9). Higher EEs of insulin in NCL obtained by the DRV method compared to CF method can be explained on the basis of structure. Hydrated lipid films (CF method) produce liposomes from lipid in a random matrix, which must form closed vesicles before encapsulation occurs. The DRV method, on the other hand, produces highly organized membrane structures of the lipids in a dried state which, after addition of aqueous solution spontaneously hydrates as closed vesicles with high EE [227]. Using the DRV method liposomes formed in drug solution are often freeze-dried, believed to enhance the EE due to intimate contact achieved between lipid and solute in dry state. However, preparation of insulin liposomes by the DRV method by addition of insulin solution to empty freeze-dried liposomes prevented the protein from any possible decomposition during the freeze-drying process and resulted in only a slight decrease in the EE (Table 4.2).

Lower EE of insulin in CM-chitin-coated liposomes may be due to replacement of the negatively-charged drug from the surface of liposomes by the polymer which also carries a negative charge at pH 7.4. Comparing the value of 8.3% associated with the outer layers of liposomes (Table 4.1) with 7% difference between insulin EEs in uncoated and CM-chitin-coated liposomes we may conclude that most of the insulin at liposomal

surface was replaced by CM-chitin. In contrast, CHM-coated liposomes favored higher EEs than the same uncoated liposomes (Figure 4.4). Since polymers were added to liposomes before removal of free drug, coating liposomes by CHM seemed to prevent loss of insulin, which is entrapped mainly in the inner lamellars of the vesicles, during frequent washing and centrifugation processes for removal of free drug. Lower EEs of insulin in uncoated than in CHM-coated liposomes reveals that a fraction of entrapped drug is lost during washing stages.

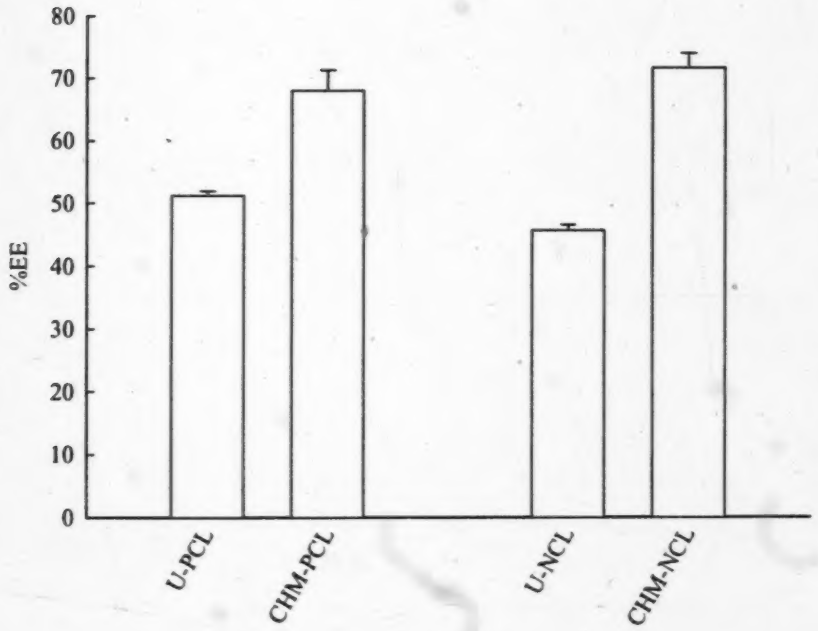


Figure 4.4. Comparison of insulin EE in uncoated and CHM-coated-PCL and NCL prepared by the DRV method. Means ± SE, n=3 (values extracted from Table 3.2).

Table 4.1. Comparison of insulin encapsulated or associated with the outer layer of preformed empty PCL (DPPC:CH:SA, 7:2:1 m.r.) prepared by the CF method[a].

Percent Encapsulation	Percent Outer Layer Association
50.7 ± 3.2[b]	8.3 ± 1.3

a. Means ± SE, n=3

b. obtained from Table 3.2

Table 4.2. Comparison of insulin EE (%) in DPPC:CH:DMPG (7:2:0.5 m.r.) liposomes prepared by the DRV method where insulin solution was introduced to empty SUVs before or after freeze-drying[a]

Before Freeze-drying	After Freeze-drying
53.27±2.36 (n=6)	45.6±0.95[b] (n=3)

a. Means ± SE

b. obtained from Table 3.2

4.3.3. Size Study

The size of phospholipid vesicles can be affected by many factors such as the preparation technique, the lipids used and the composition of the solution [228]. Increasing the mole ratio of charged adducts, i.e. SA or DMPG or DPPG, resulted in decreasing sizes of liposomes (Figures 3.10 and 3.11). The presence of either a negative or positive charge on liposomes caused electrostatic repulsion of liposome, in contrast to the tendency towards aggregation of neutral liposomes. The smaller sizes of PCL prepared by the CF method than the same liposomes prepared by DRV method seemed to be due to the mild sonication applied to the final liposomal suspension prepared by the CF method. In contrast, smaller sizes of NCL prepared by DRV than those prepared by the CF method could be again attributed to the use of sonication, to produce SUVs before freeze-drying, in DRV method while no sonication was used in CF for NCL production. No change in the size of liposomes ranging from 1-3 μm upon interaction with CHM was observed (Table 3.3), since polysaccharides with hydrophobic anchor were shown to increase the bilayer thickness by only about 1 nm [214]. This is also in agreement with Iwamoto *et al.* [229] who found not much change in the size of microemulsion droplets after coating with polysaccharide derivatives. An increase in the size of PCL coated with CM-chitin was likely due to the formation of liposome aggregates. Another possibility is aggregation of liposomes as a result of the interaction of PCL with oppositely-charged polymer. In any case no increase in the size of individual vesicles due to interaction with the polymers could be concluded.

4.3.4. Differential Scanning Calorimetric Studies of Liposomes

Differential scanning calorimetry (DSC) has been widely used to obtain values of phase transition temperature (T_m), the temperature at which the maximum peak occurs, i.e. the transition is half completed, enthalpy of the transition ($\Delta H°$), and, hence, entropy change of the transition ($\Delta S°$) of various lipid systems.

Liposomes composed of DPPC, as the only constituent, had a sharp transition mid-point T_m of 41.3 (Table 3.4). This value was lower than the values of 43, 44, 44.58 reported by Ikeda and coworkers [230], Boggs and co-workers [231] and Langer and co-workers [232]; but in good agreement with the value of 41.5 and 41.3 obtained by Papahadjopoulos and co-workers [233] and Lo and Rahman [234], and slightly higher than the value of 40.5 reported by Seki and Tirrell [235]. These differences may be attributed to various factors related to instrument or sample variability [236]. Sensitivity of the DSC instrument plays an important role [234] in that obtaining more reproducible results from high-sensitivity DSC compared to conventional DSC may be due to the nature and number of standards used to calibrate the instrument. Where conventional DSC uses indium which has a high T_m of 156.6, and therefore is more suitable for compounds with melting points above 100 °C, the high-sensitivity DSC uses two calibration standards with melting points of 28.2 and 75.9, which is more suitable for compounds of melting points below 100 °C. The Seiko DSC, model SSC/5200, allowed multiple calibration standards, advantageous in producing more reliable measurements. With regards to the heating, an increase in heating rate increases the onset and midpoint of transition temperatures [230,236,237]. Another important factor is sample

characteristics. In the case of DPPC liposomes it has been shown that where they were prepared as multilamellar vesicles (MLVs) a T_m of 41.5 [238] or 41.3 [239] was obtained, while in the form of small unilamellar vesicles (SUVs) the T_m was shifted to almost 4°C lower temperatures [238,239]. Therefore the variation in aggregation state of liposomes may be partly responsible for the differences in T_m previously mentioned.

The T_m of DPPC decreased from 41.3 to 40.1 upon inclusion of 6.7 mole% DMPG. Since the acyl chains of these phospholipids are not identical, two transitions of gel-to-liquid crystalline states were expected to occur somewhere between two T_m values. However, a DMPG peak was not detected because of low content in the mixture. The shifting of T_m of DPPC, upon incorporation of 12.5 mole% of SA, to 44.6 °C can be explained by the presence of longer acyl chains (i.e. stearyl) in the bilayers. It also caused broadening of the transition peak, indicating a lower chain cooperativity in the bilayer.

The thermal behavior of the biomembrane phase transition is affected by presence of foreign molecules. A study was conducted to evaluate the thermodynamic changes of neutral, negatively-charged, or positively-charged liposomes composed of DPPC (as the main phospholipid in the formulation) upon their interaction with polymers or insulin. Interactions between macromolecules (peptides) and phospholipids have been classified by Papahajopoulos et al. into three major groups: (1) surface adsorption only; (2) partial embedding into the lipid bilayer; and (3) penetration into the core of anionic or zwitterionic lipid bilayers [238]. The first group interaction resulted in an increase in the enthalpy of transition ($\Delta H°$) accompanied by either an increase or no change in the T_m, while processes of the second and third class resulted in reduction in the transition

enthalpy with a decrease or no change in T_m. Some contradictions to Papahajopoulos et al.'s classification scheme have been reported [234,240,241] and some modifications have been suggested [234].

Insulin only slightly reduced the enthalpy of transition in the amount incorporated (Table 3.4 and Figure 4.5) while it had no effect on the Tm of DPPC of PCL or NCL. The stabilization effect of cholesterol-derivatized polysaccharides on phospholipid bilayers has been already demonstrated using surface potential and surface pressure measurements [242]. The functionalized polysaccharides have also been shown to lower the total enthalpy and entropy changes of phospholipid after interaction and yield more thermodynamically stable systems [243]. The $\Delta H°$ of DPPC liposomes was found to be 48.65 mJ/mg (Table 3.4), in agreement with a value of 8.7 Kcal/mole (~49.55 mJ/mg) reported elsewhere [244]. Interaction of CHM with neutral DPPC liposomes decreased $\Delta H°$ from 48.65 to 41.5 mJ/mg, a 14.7 % reduction (Table 3.4 and Figure 4.5). The same pattern was also observed after CHM interaction with both PCL and NCL consisting of DPPC:SA (7:1 m.r.) and DPPC:DMPG (7:0.5 m.r.) respectively (Figure 4.5). Whereas the T_m was insensitive to such interactions. Similar thermotropic behaviors have also been reported for DPPC and DMPG upon interaction with alginate and α-sarcin, respectively [232,245]. On this basis it is suggested that CHM interacts with liposomes according to Papahadjopoulos et al. (1975) [238], i.e. the polymer becomes inserted in the lipid membrane.

In contrast to CHM, CM-chitin interactions with neutral or negatively-charged liposomes did not alter $\Delta H°$ from which it is assumed that such interactions were weak.

However, CM-chitin slightly increased ΔH° of positively-charged liposomes. This may be attributed to a stronger association of PCL with the oppositely-charged CM-chitin at pH 7.4. CM-chitin interaction with liposomes therefore falls into the first category in which macromolecules interacting with bilayers exhibit a strong dependence on electrostatic binding without significant penetration into the hydrocarbon region.

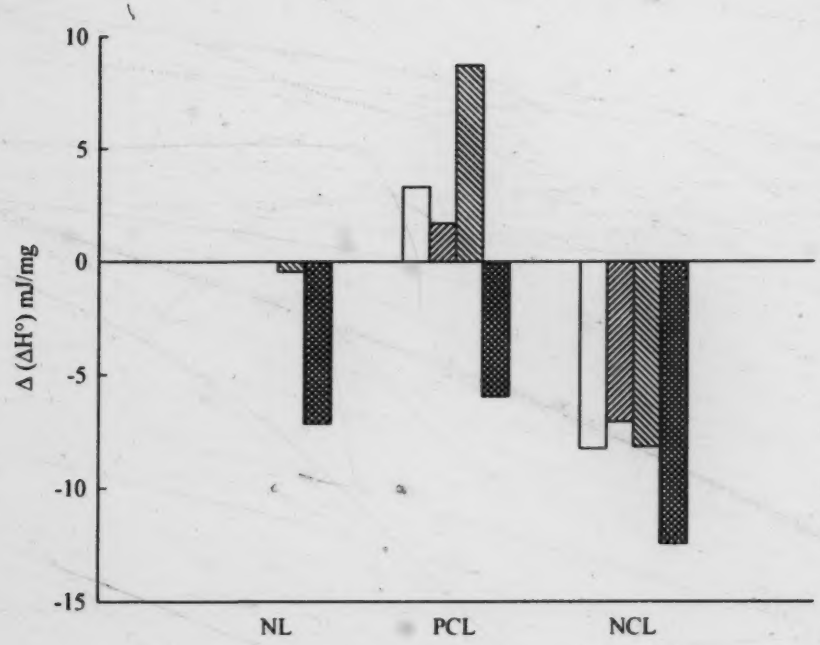


Figure 4.5 The effect of insulin incorporation or polymer-coating on the enthalpy change (ΔH°) of the main transition endotherms of neutral liposomes (NL), PCL, and NCL composed of DPPC, DPPC:SA (7:1 m.r.), and DPPC:DMPG (7:0.5 m.r.) respectively. For the sake of comparison, the enthalpy changes of the transitions of all systems were subtracted from that of liposomes composed of DPPC only (horizontal line at zero). Values obtained from Table 3.4. ▭, empty and uncoated; ▨, insulin-encapsulated and uncoated; ▩, CM-chitin-coated; and ▩, CHM-coated.

4.4. Liposome Behavior in Various Media

4.4.1. Stability in bile salt solutions

Detergent - phospholipid bilayers interaction results in the breakdown of lamellar structure, formation of lipid-detergent mixed micelles, and release of entrapped drugs [215,216]. The first stage of interaction is fast and one may observe an increase in turbidity which reflects association of detergent with the bilayer which increases vesicle weight with only a slight increase of vesicle radius. The second stage, lamellar-micellar phase transition, is normally associated with a decrease in turbidity which is interpreted as bilayer solubilization starting with the outer bilayer and proceeding through the remaining ones (slow stage) [219]. Little or no change in turbidity of CHM-PCL or U-PCL in solutions of up to 10 mM SC at 37 °C (Figure 3.19) can be interpreted as association of the bile salt with the bilayer without any important changes in permeability of vesicles and a decrease in turbidity at SC concentrations above 10 mM as the result of solubilization of liposomes. In the case of CM-chitin-PCL, solubilization began at lower SC concentration of 2 mM (Figure 3.19). This seems to have resulted from faster saturation of bilayers by detergent molecules which is reflected in the leakier nature of theses liposomes caused by CM-chitin coating. A plateau stage of turbidity above 6 mM SC might, however, be attributed to prevention of further penetration of cholate anions due to electrostatic repulsion by carboxyl groups of CM-chitin. However, one should bear in mind that the electrostatic interaction between the surfactant and the liposome only modulate the permeability alterations in the lipid bilayer, the hydrophobic interaction being the main factor involved in the modification of these parameters [220]. Liposomes

of either charge, coated or uncoated, resisted disruption by SC concentrations from 0-24 mM at RT resulting in great stability (Figures 3.18; 3.20). The rigidity of gel-state DPPC bilayers or to the presence of a high level of CH (> 20% m.r.) slowed the solubilization rate [98]. Slightly lower turbidities of CM-chitin-coated liposomes (Figures 3.18; 3.20) may again be due to the leakier nature of the bilayers, as discussed earlier.

4.4.2. Stability of insulin in liposomes in the presence of enzymes

Pepsin, trypsin, and α-chymotrypsin are the most abundant luminal proteolytic enzymes in the GI tract. Liposomes have been found to protect their insulin content against enzymatic degradation (Figure 3.21) while free insulin was almost completely degraded under the same conditions. It may be concluded that liposomes prevented enzymes accessing entrapped insulin. Moreover, enzymes themselves were likely associated with the surfaces of bilayers resulting in reduced proteolytic activity [114]. The protective effect of the CHM coating of liposomes in pepsin seemed to be slightly higher than that of uncoated liposomes because of less leakage of insulin and/or steric hindrance caused by the polymer. Likewise, better protection of insulin encapsulated in CHM-liposomes in α-chymotrypsin than in pepsin solutions seemed to be because of lower leakage of insulin from liposomes at pH 7.8 than at pH 2 if the extent of ^{125}I-insulin release in SGF (pH 2) and SIF (pH 7.4) (Figures 3.25 and 3.27) are compared.

4.4.3. Release of insulin

Kinetics of release of drugs from liposomes have been suggested to follow zero, first, second, or any non-integer rate order depending on size, charge, hydrophobicity or hydrophilicity of the solute entrapped, liposome size, charge, type, rigidity/fluidity, and environmental factors such as temperature, pH, ionic strength and chemical content of the medium [134,135]. A two-compartment first order kinetic model has been also proposed [138-140].

Biphasic kinetic release of insulin from liposomes are shown in Figures 3.22-3.31. The initial rapid phase, which lasted about 1-2 h, was likely due to the desorption of adsorbed insulin from the surface and also to its leakage from the outer layers of vesicles. The second slower phase seemed to be due primarily to the diffusion of insulin through the bilayers [140]. Insulin release rates from CM-chitin-PCL in SGF or SIF were essentially not different than that from U-PCL (Figures 3.22, 3.24). Negatively-charged CM-chitin may stabilize PCL by electrostatic binding, giving rise to a very slow release in pH 7.4 PBS solution. However, it can not adequately protect the liposome and insulin content against destructive effects of digestive contents of SIF which can be concluded from comparing the release rates in Figure 3.23. This might have arisen from the fact that CM-chitin, lacking a hydrophobic moiety, did not penetrate into the bilayer and was desorbed from the surface of vesicles upon dilution or by other constituents of SIF competing with polymer for adsorption at liposome surfaces. Such compounds are absent in PBS solution. In contrast, CHM-coated liposomes of both charge were found to have reduced insulin leakage in SIF (Figures 3.24, 3.27, 3.28 & 3.30). This is believed to be

resulted from the liposome stabilization by CHM via anchorage of its hydrophobic moiety in the bilaye, as was also evident from DSC studies (Figure 4.5). A relatively higher release rate of insulin from U-NCL in SIF than in SIF without enzyme may be attributed again to the destructive effect of enzymes and resisted the hostile conditions (Figure 3.28). However, the effect of CHM was undermined in SGF by a very steep pH gradient inside liposomes (pH 7.4) and the equilibrium medium (pH 2) (Figures 3.25; 3.26). Release study of insulin as a function of liposome composition is depicted in Figures 3.29 & 3.30. The slower release rate of insulin from DSPC liposomes in both SGF and SIF without enzyme which can be attributed to the greater rigidity of the bilayer(T_m = 53 °C) (cf. T_m of DPPC= 41.4 °C).

4.5. In Vivo Studies

Different methods of glucose analysis have been developed and applied. Ease, convenience, and speed of manual glucose measurement apparatuses with reagent test strips have attracted not only ordinary diabetic persons to regularly check their blood glucose levels but also many investigators to quantify the blood glucose levels of subjects reported in the scientific literature [246-249]. Deviations observed in the calibration curve of blood glucose by reagent test strips with a Blood Glucose Sensor Electrodes apparatus (Medisense®, England) from that determined by an Astra-8® analyzer (Beckman® Instruments Inc., USA) cautions for a more careful interpretation of the results. As seen in Figure 3.32, linear correlation between blood glucose concentrations and the responses of both instruments were obtained, however, underestimation of blood glucose, especially at

higher concentrations, may happen when using the manual glucose-meter apparatus (Medisense®). Another concern regarding their use for quantification purposes is their quite high inter-variability and sensitivity to amount of blood used. Methodologic variability is inherent with the test strip. For instance, as plasma is absorbed by the strip from the drop of whole blood, hemoconcentration of cells, protein, and other less permeable constituents occurs at the test strip surface, blocking further diffusion. The reagent strip, therefore, measures the glucose content of the absorbed fluid rather than glucose concentration of the samples [250]. A discrepancy (> 15% difference) between some hand-held glucose-meters and laboratory results in 27-34% of the times has been reported [251]. Therefore, a manual glucose-meter (Medisense®) was used for early screening of diabetes development in animals only, but all blood samples of treatment groups were analyzed by a glucose oxygenase method using an Astra-8® analyzer.

The control groups receiving PBS, empty liposomes or insulin solution (400 U/kg) exhibited quite similar blood glucose profiles, i.e. minor fluctuations around the base line. However, insulin solution seemed to decrease the blood glucose very slightly but not significantly different from PBS or empty liposomes groups). This may be attributed to a very low oral bioavailability of insulin solution which has also been reported by other investigators [252]. In contrast, administration of empty liposomes elevated the blood glucose in early hours after intragastric administration. This is likely because of the activation of glucogenesis cycle upon absorption of lipids. A glucogenic effect of carriers has also been observed by others with liposomes [252] and microemulsions [253]. Quick

absorption and relatively high bioavailability of insulin after s.c. injection was predictable as was evident from the hypoglycemic profiles of rats as depicted in Figure 3.34.

The stronger hypoglycemic effects of CHM-NCL than U-NCL (Figure 3.35) can be attributed to their higher thermodynamic stabilities (Table 3.4 and Figure 4.5), better performance in protecting the insulin content in an enzymatic environment (Figure 3.21), and providing less leakage of insulin in SGF and SIF (Figure 3.25-3.28). Also a close contact of the formulation to the absorptive surface of GI tract due to coverage of liposome surface by CHM, a relatively high molecular weight polymer with swelling capacity, as well as the ability to form hydrogen bonds, may result in a reduced luminal clearance and a steeper concentration gradient of insulin. Insulin-U-NCL showed a relatively lower hypoglycemic effect than insulin-U-PCL (Figure 3.37a). Insulin (pI = 5.6), being negatively-charged at pHs above the pI, may repulse the negatively-charged liposomes. Therefore, the head of phospholipid may be twisted which results in lower contact with the intestinal membrane [248]. The results are in agreement with Li & Mitra (1995) [248] who had similar observations for pulmonary delivery of insulin liposomes. Another possibility is the stronger affinity of positively-charged liposomes to GI absorptive surface, being overall negatively-charged. Such a mechanism has been proposed for stronger adhesion of positively-charged liposomes, compared to neutral or negatively-charged liposomes, to the mucinous epithelial surfaces in the eyes [254]. Similarly, a stronger interaction of U-PCL with GI tract may also partly explain its unexpected slightly higher hypoglycemic effect than CHM-PCL (Figures 3.36 & 3.40), in which the positive charge of vesicles are suppressed by the polymer coated on the surface,

although the real reason is not known and might not be that simple, as is the case when comparing CHM-PCL with CHM-NCL (Figures 3.37.b; 3.40). The pharmacologic effect ($AAC_{0\text{-}12h}$ of glucose) was found to be proportional to the log of the dose of CHM-NCL containing insulin with a reasonably good correlation-coefficient of $r^2 > 0.95$ (Figure 3.39). This was, perhaps, the most striking characteristic of this insulin delivery system and may imply that a predictable pharmacologic effect upon oral delivery of CHM-NCL containing insulin can be achieved.

5. SUMMARY

The present studies have indicated that reproducible cholesterol derivatization of polysaccharides could be achieved upon modification of carboxymethylation step of preparation procedure (see Table 3.1 and section 4.1 for the details).

Such polymers (like CHM) interacted more strongly than a polymer lacking a hydrophobic moiety (i.e. CM-chitin) with liposomal bilayers. This was evident from coating efficiency studies of liposomes revealing a higher CE of liposomes by CHM than by CM-chitin (Figure 3.6), and also from the DSC study indicating penetration of CHM by its hydrophobic moiety, but not of CM-chitin, into the bilayer (Figure 3.16; Table 3.4).

Relatively high entrapment of insulin in liposomes using mild conditions was obtained using an appropriate composition and preparation method. High EE of insulin in PCL, by the CF or DRV method, was the result of electrostatic association of insulin carrying a negative charge at pH 7.4 with oppositely-charged liposomes (Figure 3.7; Table 3.2), whereas a high EE in NCL prepared by the DRV method resulted from use of a highly-concentrated insulin solution to highly-organized lipid membrane structures (Table 3.2). Interestingly, CHM-coated liposomes resulted in higher EEs than the same uncoated liposomes (Figure 4.4) because CHM retarded loss of insulin during the washing steps.

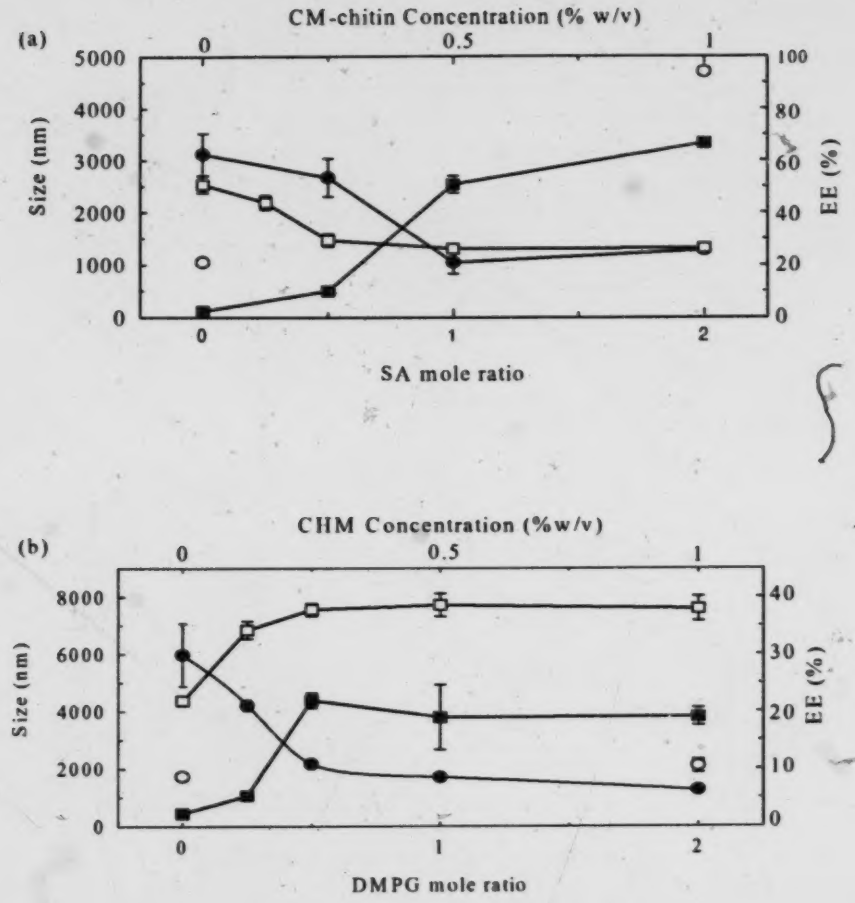


Figure 5.1. Optimization of the EE of insulin (square) and the size (circle) of (a) DPPC:CH:SA (7:2:X m.r.) or (b) DPPC:CH:DMPG (7:2:Y m.r.) liposomes prepared by the CF method as a function of SA or DPMG (closed symbols) or polymer concentration (open symbols). Notes: 1) Effects of polymer concentrations were evaluated at fixed SA or DMPG mole ratios of 1 and 0.5, respectively. 2) Plots are extracted from **RESULTS** and re-arranged.

Inclusion of charged adducts not only improved the EE of insulin but, at the same time, reduced the liposome size (Figure 5.1). This is a desirable feature since liposomes of smaller sizes have a larger interacting surface area for reacting with polymers and the walls of the GI tract.

Turbidity studies of liposomes in SC solutions of 0-24 mM, indicated excellent stability at RT over the whole range of concentration seemingly because of the rigidity of gel-state DPPC bilayers and/or presence of relatively high level of cholesterol, whereas at 37 °C a reduced stability was observed above 10 mM SC concentrations. Liposome stability was essentially not affected by CHM-coating whereas CM-chitin increased liposome (instability) (Figures 3.18-3.20).

Release studies revealed that CHM-coated liposomes reduced leakage of insulin in SIF (Figures 3.24, 3.27, 3.28, 3.30), presumably due to improved stability of membranes as was evident from DSC studies (Figure 4.6), and also from better protection of entrapped insulin against proteolytic enzymes (Figure 3.21). CM-chitin was not as effective. SGF undermined the effect of polymer-coating on liposomes. DSPC liposomes showed slower release rates than DPPC liposomes because of higher bilayer rigidity (Figures 3.29-3.30).

Finally, in vivo studies demonstrated a higher hypoglycemic effect of CHM-NCL among all formulations because of lower insulin leakage, better stability in GI fluids, and presumably a greater retention of liposomes at the absorptive surface of GI tract due to CHM coverage. The most striking finding was the proportionality of the hypoglycemic effect to the log of the dose with a reasonably good correlation coefficient of $r^2 > 0.95$

(Figure 3.39), indicating the potential of polymer-coated liposomes for oral delivery of insulin (or other peptides), and, the possibility of predictability of diabetic blood glucose after oral delivery of CHM-NCL insulin.

6. CONCLUSIONS

1. Cholesterol derivatization of a polysaccharide has been improved in the carboxymethylation step after reacting polysaccharide with NaH in DMSO and bromomethylacetate followed by hydrolysis in alkaline medium (cf. Inman, 1975; Sato, 1990).

2. Low amounts of CM-chitin and CH-derivatized polysaccharides derived from the surface of liposomes (as low as 1 μg/ml) were quantified by a simple turbidimetric method.

3. Association and anchoring of CHM, a hydrophobized polymer to the bilayer yielded greater CE than CM-chitin (no anchor) (10 percent versus 3 percent).

4. DSC studies indicated that polymer association caused no change in T_m but the changes in the enthalpy of transition ($\Delta(\Delta H°)$) supported an anchoring mechanism of CHM to the bilayers but no changes occurred with CM-chitin suggesting no significant interaction.

5. Relatively high insulin EE (20 - 67 percent) was obtainable in liposomes under mild conditions as a function of lipid composition and preparation technique.

6. Liposome size was not changed by polymer-coating. although the apparent larger average size of positively-charged liposomes coated with CM-chitin compared to uncoated liposomes was possibly caused by aggregation.

7. CM-chitin-coating of positively-charged liposomes decreased EE, reduced stability in SIF and increased rate of insulin release.

8. Liposomes offered protection of insulin against proteolytic enzymes in solution.

9. Liposomes coated with CHM were superior to the other formulations in reducing insulin leakage rate. Accordingly, a greater hypoglycemic effect was obtained from CHM-NCL compared to NCL, PCL, and CHM-PCL.

10. Overall, in vitro and in vivo studies indicated that coating liposomes with CHM enabled more insulin to be absorbed from the GI tract than would otherwise be the case.

7. FUTURE WORK

The results of this study reveals the potential of polymer-coated liposomes for oral delivery of insulin. However, more detailed studies have yet to be done to reveal the mechanisms of drug absorption and further improvement of the system is required to boost insulin absorption efficiency and the timing requirements.

REFERENCES

[1] Banerjee, P.S., Hosny, E.A. & Robinson, J.R. (1991) Pareteral Delivery of Peptide and Protein Drugs. In: *Peptide and Protein Drug Delivery*, pp. 487-543. Ed. Lee, V.H.L. Marcel Dekker, INC., New York.

[2] Lee, V.H.L. (1991) Changing needs in drug delivery. In: *Peptide and protein drug delivery*, pp. 1-56. Ed. Lee, V.H.L. Marcel Dekker, New York.

[3] Lee, V.H.L. (1986) Peptide and protein drug delivery: opportunities and challenges. *Pharm.Int.*, 7, 208-212.

[4] Samanen, J. (1985) Biomedical polypeptides, a wellspring of pharmaceuticals. In: *Bioactive Polymeric Systems*, pp. 279-344. Ed. Gebelein, C.G. & Carraher, C.E. Plenum Press, New York.

[5] Barnhart, E.R. (1988) *Physician's Desk Reference*. Medical Economics Company, Oradell, N.J.

[6] Schilling, R.J. & Mitra, A.K. (1990) Intestinal mucosal transport of insulin. *Int.J.Pharm.*, **62**, 53-64.

[7] Lee, V.H.L., Traver, R.D. & Taub, M.E. (1991) Enzymatic barriers to peptide and protein drug delivery. In: *Peptide and protein drug delivery*, pp. 303-358. Ed. Lee, V.H.L. Marcel Deker Inc., New York.

[8] Hooper, N.M. (1993) Ectopeptidases. In: *Biological barriers to protein delivery*, Ed. Audus, K.L. & Raub, T.J. Plenum Press, New York.

[9] Lee, V.H.L. & Yamamoto, A. (1990) Penetration and enzymatic barriers to peptide and protein absorption. *Adv.Drug Del.Rev.*, **4**, 171

[10] Palmieri, F.E. & Ward, P.E. (1983) Mesentery vascular metabolism of substance P. *Biochim.Biophys.Acta*, **755**, 522-525.

[11] Palmieri, F.E., petrelli, J.J. & Ward, P.E. (1985) Vascular, plasma membrane aminopeptidase M.: Metabolism of vasoactive peptides. *Biochem.Pharmacol.*, **34**, 2309-2317.

[12] Najdovski, T., Collette, N. & Descholt-Lankman, M. (1984) Hydrolysis of C-terminal octapeptide of cholecystokinin by rat kidney membranes: chracterization of the cleavage by solubsilized endopeptides-24.11. *Life Sci.*, **37**, 827-834.

[13] Dodda Kashi, S. & Lee, V.H.L. (1986) Enkephalin hydrolysis in homogenates of various absorptive mucosae in the albino rabbite.: similarities in rates and invovement of aminopeptidases. *Life Sci.*, **38**, 2019-2028.

[14] Lee, V.H.L., Carson, L.W., Kashi, S.D. & Stratford, R.E., Jr. (1986) Metabolic and permeation barriers to the ocular absorption of topically applied enkephalins in albino rabbits. *J.Ocular.Pharmacol.*, **2**, 345-352.

[15] Lee, V.H.L., Dodda-Kashi, S., Grass, G.M. & Rubas, W. (1991) Oral route of peptide and protein drug delivery. In: *Peptide and protein drug delivery*, pp. 691-738. Ed. Lee, V.H.L. Marcel Dekker, INC., New York.

[16] Baker, J., Hidalgo, I.J. & Borchardt, R.T. (1991) Intestinal epithelial and vascular endothelial barriers to peptide and protein delivery. In: *Peptide and protein drug delivery*, pp. 359-390. Ed. Lee, V.H.L. Marcel Dekker Inc, New York.

[17] Madara, J.L. (1989) Loosening tight junctions: lessons from the intestine. *Clin.Invest.*, **83**, 1089-1094.

[18] Madara, J.L. (1988) Tight junction dynamics: is paracellular transport regulated?. *Cell*, **53**, 497-498.

[19] Neutra, M.R. & Kraehenbuhl, J.-P. (1993) Transepithelial transport of proteins by intestinal epithelial cells. In: *Biological barriers to protein delivery*, pp. 107-129. Ed. Audus, K.L. & Raub, T.J. Plenum Press, New York.

[20] Cornes, J.S. (1965) Number, size, and distribution of peyer's patches in the human small intestine. Part I. The development of peyer's patches. *Gut*, **6**, 225-233.

[21] Neutra, M.R. & Kraehenbuhl, J.P. (1992) Transepithelial transport and mucosal defense. *Trendse Cell Biol.*, **2**, 134-138.

[22] Edwards, H.H., Muller, T.J. & Morrison, M. (1979) Distribution of transmembrane polypeptides in freeze fracture. *Science*, **203**, 1343-1346.

[23] Eicholz, A. (1968) Studies on the organization of the brush border in intestinal epithelial cells V. Subfraction of enzymatic activities of the microvillus membrane. *Biochim.Biophys.Acta*, **163**, 101-107.

[24] Bouhours, J.-F. & Glickman, R.M. (1977) Rat intestinal glycolipids. III. Fatty acids and long chain bases of glycolipids from villus and crypt cells. *Biocim.Biophys.Acta.*, **487**, 51-60.

[25] Kawai, K., Fujita, M. & Nakao, M. (1974) Lipid components of two different regions of an intestinal epithelial cell membranes of mouse. *Biocim.Biophys.Acta.*, **369**, 222-233.

[26] Douglas, A.P., Kerley, R. & Isselbacher, K.J. (1972) Preparation and characterization of the lateral and basal plasma membranes of the rat intestinal epithelial cell. *Biochemistry*, **128**, 1329-1338.

[27] Schachter, D. & Schinitzky, M. (1977) Fluorescence polarization studies of rat intestinal microvillus membranes. *Clin.Invest.*, **59**, 536-548.

[28] Swenson, E.S. & Curatolo, w.J. (1992) Means to enhance penetration: (2) Intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity. *Adv.Drug Del.Rev.*, **8**, 39-92.

[29] Brambell, F.W.R., Haliday, R., Brierley, R. & Hemmings, W.A. (1954) Transference of passive immunity from mother to young. *Lancet*, **1**, 694-695.

[30] Siminoski, K., Gonella, P., Bernanke, J., Owen, L., Neutra, M.R. & Murphy, R.A. (1986) Uptake and transepithelial transport of nerve growth factor in suckling rat ileum. *J.Cell Biol.*, **103**, 1979-1990.

[31] Thornburg, W., Matrisian, L., Magnun, B. & Koldousky, O. (1984) Gastrointestinal absorption of epidemal growth factor in suckling rats. *Am.J.Physiol.*, **246**, 680-685.

[32] Rao, R.K., Thornburg, W., Korc, M., Matrisian, L.M., Magun, B.E. & Koldovsky, O. (1986) Processing of epidermal growth factor by suckling and adult rat intestinal cells. *Am.J.Physiol.*, **250**, G850-G855

[33] Gallo-Payet, N. & Hugon, j.s. (1985) Epidermal growth factor receptors in isolated adult mouse intestinal cells: studies in vivo and in organ culture. *Endocrinology*, **116**, 194-201.

[34] Thompson, J.F. (1986) Evidence of specific epidermal growth factor (EGF) receptors on fetal, suckling, and adult rat intestinal microvillus membrane (MVM). *Gasteroenterology*, **90**, 1664

[35] Gonella, P., Murphy, R., Owen, L., Bernanke, J. & Neutra, M. (1984) Epidermal growth factor (EFG) is transported across the epithellium of suckling rat ileum. *J.Cell Biol.*, **99**, 283a(Abstract)

[36] Cerejido, M., Ruiz, O., Gonzalez-Mariscal, L., Contreras, R.G., Balda, M.S. & Garcia-Villegas, M.R. (1993) The Paracellular Pathway: A Small Version of the Kidney Nephron. In: *Biological Barriers to Protein Delivery*, pp. 3-22. Ed. Audus, K.L. & Raub, T.J. Plenum Press, New York.

[37] Lundin, S. & Artursson, P. (1990) Absorption of a vasopressin analogue, 1-deamino-8-D-arginine-vasopressin (dDAVP), in a human intestinal epithelial cell line, Caco-2. *Int.J.Pharm.*, **64**, 181-186.

[38] Thwaites, D.T., Hirst, B.H. & Simmons, N.L. (1993) Passive transepithelial absorption of thyrotropin-releasing hormone (TRH) via a paracellular route in cultured intestinal and renal epithelial cell lines. *Pharm.Res.*, **10**, 674-681.

[39] Drew, J., Fricker, G., Vonderscher, J. & Beglinger, C. (1993) Enteral absorption of octrotide: absorption enhancement by poloxyethylene-24-cholesterol ether. *Br.J.Pharmacol.*, **108**, 298-303.

[40] Jaehde, U., Masereeuw, R., de Boer, A.G., Fricker, G., Nagelkerke, J.F., Vonderscher, J. & Briemer, D.D. (1994) Quantification and visualization of the transport of octreotide, a somatostatin analogue, across monolayers of cerebrovascular endothelial cells. *Pharm.Res.*, **11**, 442-448.

[41] Pauletti, G.M., Gangwar, S., Knipp, T., Neurarkar, M.M., Okumu, F.W., Tamura, K., Siahaan, T.J. & Borchardt, R.T. (1996) Structural requirements for intestinal absorption of peptide drugs. *J.Control.Rel.*, **41**, 3-17.

[42] Okumu, F.W., Pauletti, G.M., Vander Velde, D.G., Siahaan, T.J. & Borchardt, R.T. (1995) The effect of conformational flexibility on the permeability of a hexapeptide across monolayers of a cultured human intestinal epithelial cell (Caco2 cells). *Pharm.Res.*, **12**, S-302

[43] Adson, A., Raub, T.J., Burton, P.S., Barsuhn, C.L., Hilgers, A.R., Audus, K.L. & Ho, N.F.H. (1994) Quantitaive approaches to delineate paracellular diffusion in cultured epithelial cell monolayers. *J.Pharm.Sci.*, **83**, 1529-1536.

[44] Rubas, W., Cromwell, M., Gadek, T., Narindray, D. & Mrsny, R. (1994) Structural elements which govern the resistance of intestinal tissues to compound transport. *Mat.Res.Soc.Symp.Proc.*, **331**, 179-185.

[45] Rubas, W., Villagran, J., Cromwell, M., McLeod, A., Wassenberg, J. & Mrsny, R. (1995) Correlation of solute flux across Caco-2 monolayers and colonic tissue in vitro. *S.T.P.Pharm.Sci.*, **5**, 93-97.

[46] Knipp, G.T., Vander Velde, D.G., Siahaa, T.J. & Borchardt, R.T. (1995) The effect of solution and charge on the paracellular permeability of model pentapeptides across Caco-2 cell monolayers. *Pharm.Res.*, **12**, S-303

[47] Kahns, A.H., Buur, A. & Bundgaard, H. (1993) Prodrugs of peptides.18. synthesis and evaluation of various esters of desmopressin (dDAVP). *Pharm.Res.*, **10**, 68-74.

[48] Muranishi, S., Murakami, M., Hashidzume, M., Yamada, K., Tajima, S. & Kiso, Y. (1992) Trials of lipid modifications of peptide hormones for intestinal delivery. *J.Control.Rel.*, **19**, 179

[49] Jennewein, H.M., Waldeck, F. & Konz, W. (1974) The absorption of tetragastrin from different sites in rats and dogs. *Arzneim.Forsch./ Drug Research*, **24**, 1225-1228.

[50] Takaori, K., Burton, J. & Donowitz, M. (1986) The transport of an intact oligopeptide across adult mamalian jejunum. *Biochem.Biophys.Res.Commun.*, **137**, 682-687.

[51] Hussain, M.A., Shenvi, A.B., Rowe, S.M. & Shefter, E. (1989) The use of alpha-aminobrobic acid derivatives to stabilize peptide drugs during their intranasal absorption. *Pharm.Res.*, **6**, 186-189.

[52] Lundin, P.D.P., Lundin, S., Olsson, H., Karlsson, B.W., Westrom, B.R. & Pierzynowski, S.G. (1995) Enhanced intestinal absorption of oxytocin analogues in the absence of pancreatic juice in pigs. *Pharm.Res.*, **12**, 1478-1482.

[53] Wuthrich, MP., Martenet, M. & Buri, P. (1994) Effect of formulation additives upon the intranasal bioavailability of a peptide drug: tetracosactide (ACTH1-24). *Pharm.Res.*, **11**, 278-282.

[54] Langguth, P., Merkle, H.P. & Amidon, G.L. (1994) Oral absorption of peptides: the effect of absorption site and enzyme inhibition on the systemic availability of metkephamid. *Pharm.Res.*, **11**, 528-535.

[55] Bai, J.P.F. & Chang, L.L. (1995) Transepithelial transport of insulin: I. Insulin degradation by insulin-degrading enzyme in small intestinal epithelium. *Pharm.Res.*, **12** , 1171-1175.

[56] Bai, J.P.F., Hsu, M.J.P. & Shier, W.T. (1995) Insulin-degrading enzyme in a human colon adenocarcinoma cell line (Caco-2). *Pharm.Res.*, **12**, 513-517.

[57] Kidron, M., Bar-On, H., Berry, E.M. & Ziv, E. (1982) The absorption of insulin from various regions of the rat intestine. *Life Science*, **31**, 2837-2841.

[58] Ziv, E., Lior, O. & Kidron, M. (1987) Absorption of protein via the intestinal wall. A quantitative model. *Biochem.Pharmacol.*, **36**, 1035-1039.

[59] Hirai, S., Yashiki, T. & Mima, H. (1981) Mechanisms for the enhancement of the nasal absorption of insulin by surfactants. *Int.J.Pharm.*, **9**, 173-184.

[60] Lee, V. H. L., Dodda Kashi, S., Patel, R. H., Hayakawa, E., and Inagaki, K. Proc. Intern. Symp. Control. Rel. Bioact. Mater. 14, 23-24. 1987. Ref Type: Conference Proceeding

[61] Lee, V. H. L., Gallardo, D., and Longenecker, J. P. Proc. Intern. Symp. Control. Rel. Bioact. Mater. 14, 55-56. 1987. Ref Type: Conference Proceeding

[62] Hayakawa, E., Yamamoto, A., Shoji, Y. & Lee, V.H.L. (1989) Effect of sodium glycocholate and polyethylene-9-lauryl ether on the hydrolysis of varying concentrations of insulin in the nasal homogenates of the albino rabbit. *Life Sci.*, **45**, 167-174.

[63] Saffran, M., Bedra, C., Kumar, G.S. & Neckers, D.C. (1988) Vasopressin: a model for the study of effects of additives on the oral and rectal administration of peptide drugs. *J.Pharm.Sci.*, **77**, 33-38.

[64] Ondetti, M.A. & Cushman, D.W. (1985) Angiotensin-converting enzyme inhibitors: biochemical properties and biological actions. *Crit.Rev.Biochem.*, **16**, 381-411.

[65] Verstraete, M. (1985) Clinical application of fibrinolysis. *Drugs*, **29**, 236-261.

[66] Hussain, M.A., Koval, C.A., Shenvi, A.B. & Aungst, J. (1990) Recovery of rat nasal mucosa from the effect of aminopeptidase inhibitors. *J.Pharm.Sci.*, **79**, 398-400.

[67] Narawane, L. & Lee, V.H.L. (1994) *Drug Absorption Enhancement. Concepts, Possibilities, Limitations, and Trends*, pp. 1Ed. Boer, A.G.d. Harwood Academic, Netherlands.

[68] Sato, K., Sugibayashi, K. & Morimoto, Y. (1988) Effect and mode of action of aliphatic esters on the invitro skin permeation of nicorandil. *Intern.J.Pharm.*, **43**, 31-40.

[69] Tomita, M., Shiga, M., Hayashi, M. & Awazu, S. (1988) Enhancement of colonic drug absorption by the paracellular permeation route. *Pharm.Res.*, **5**, 341-346.

[70] Kajii, H., Horie, T., Hayashi, M. & Awazu, S. (1988) Fluorescence study of the membrane-perturbing action of sodium caprylate as related to promotion of drug absorption. *J.Pharm.Sci.*, **77**, 390-392.

[71] Murakami, M., Takada, K., Fujii, T. & Muranishi, S. (1988) Intestinal absorption enhanced by unsaturated fatty acids: inhibitory effect of sulfhydryl modifiers, *Biochim.Biophys.Acta*, **939** , 238-246

[72] Tomita, M., Hayashi, M., Horie, T., Ishizawa, T. & Awazu, A. (1988) Enhancement of colonic drug absorption by the transcellular permeation route. *Pharm.Res.*, **5**, 786-789.

[73] Freel, R.W., Hatch, M., Earnest, D.L. & Goldner, A.M. (1983) Role of tight junctional pathways in bile salt-induced increases in colonic permeability. *Am.J.Physiol.*, **245**, G816-G823

[74] Shiga, M., Hayashi, M., Horie, T. & Awazu, S. (1987) Differences in the promotion mechanism of the colonic absorption of antipyrine, phenol red and cefmetazole. *J.Pharm.Pharmacol.*, **39**, 118-123.

[75] Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M. & Roche, A.C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res.*, **21**, 871-878.

[76] Villegas, J.C. & Broadwell, R.D. (1993) Transctosis of protein through the mammalian cerebral epithelium and endothelium. II. Adsorptive trannscytosis of WGA-HRP and the blood-brain and brain-blood barriers. *J.Neurocytol.*, **22**, 67-80.

[77] Lehr, C.M. & Lee, V.H.L. (1993) Binding and transport of some bioadhesive plant lectins across Caco-2 cell membrane. *Pharm.Res.*, **10**, 1796-1799.

[78] Miura, S., Song, S.I., Morita, A., Erickson, R.H. & Kim, Y.S. (1983) Distribution and biosynthesis of aminopeptidase N and dipeptidyl aminopeptidase IV in rat small intestine. *Biochim.Biophys.Acta*, **761**, 66-75.

[79] Vaeth, G.F. & Henning, S.J. (1982) Postnatal development of peptidase enzyme in rat small intestine. *J.Pediatr.Gastroenterol.Nutr.*, **1**, 111-117.

[80] Asatoor, A.M., Chadha, A., Milne, M.D. & Prosser, D.I. (1973) Intestinal absorption of stereoisomers of dipeptides in the rat. *Clin.Sci.Mol.Med.*, **Supplement. 42**, 199-212.

[81] Michel, C., Aprahamian, M., Defontaine, P., Couvreur, P. & Damge, C. (1991) The effect of site of administration in the gastrointestinal tract on the absorption of insulin from nanocapsules in diabetic rats. *J.Pharm.Pharmacol.*, **43**, 1-5.

[82] Morishita, M., Morishita, I., Takayama, K., Machida, Y. & Nagai, T. (1993) Site-dependent effect of aprotinin, sodium caprate, Na2EDTA and sodium glycocholate on intestinal absorption of insulin. *Biol.Pharm.Bull.*, **16**, 68-72.

[83] Morishita, I., Morishita, M., Takayama, K., Machida, Y. & Nagai, T. (1993) Enteral insulin delivery by microspheres in 3 different formulations using Eudargit L100 and S100. *Int.J.Pharm.*, **91**, 29-37.

[84] Ritschel, W.A. (1991) Targeting in the gastrointestinal tract: new approaches. *Meth.Find.Exp.Clin.Pharmacol.*, **13**, 313-336.

[85] Rubinstein, A. & Tirosh, B. (1994) Mucus gel thickness and turnover in the gastrointestinal tract of the rat: response to cholnergic stimulus and implication for mucoadhesion. *Pharm.Res.*, **11**, 794-799.

[86] Bai, J.P.F. (1993) Distribution of brush-border membrane peptidases along the rabbit intestine: implication for oral delivery of peptide drugs. *Life Sci.*, **52**, 941-947.

[87] Krevsky, B., Malmud, L.S., D'Ercole, F., Maurer, A.H. & Fisher, R.S. (1986) Colonic transit scintigraphy. A physiologic approach to the quantitative measurement of colonic transit in humans. *Gastroenterology*, **91**, 1102-1112.

[88] Bauer, K.H. & Kesselhut, J.F. (1995) Novel pharmaceutical exipients for colon targeting. *S.T.P.Pharma.Sci.*, **5**, 54-59.

[89] Milojevic, S., Newton, J.M., Cummings, J.H., Gibson, G.R., Bothman, R.L., Ring, S.G., Allwood, M.C. & Stockham, M. (1995) Amylose, the new perspective in oral drug delivery to the human large intestine. *S.T.P.Pharma.Sci.*, **5**, 47-53.

[90] Gazzaniga, A., Busetti, C., Moro, L., Sangaili, M.E. & Glordano, F. (1995) Time-dependent oral delivery systems for colon targeting. *S.T.P.Pharma Sci.*, **5**, 83-88.

[91] Saffran, M., Kumar, G.S., Savariar, C., Burnham, J.C., Williams, F. & Neckers, D.C. (1986) A new approach to the oral administration of insulin and other peptide drugs. *Science*, **233**, 1081-1084.

[92] Brondsted, H. & Kopecek, J. (1991) Hydrogels for site-specific oral drug delivery: synthesis and characterization. *Biomaterials*, **12**, 584-592.

[93] Yeh, P.Y., Berenson, M.M., Samowitz, W.S., Kopeck[illegible]a, P. & Kopecek, J. (1995) Site-specific drug delivery and penetration enhancement in the gastrointestinal tract. *J.Control.Rel.*, **36**, 109-124.

[94] Lioyed, A.W., Martin, G.P. & Soozandehfar, S.H. (1994) Azopolymers: ameans of colon specific drug delivery? *Int.J.Pharm.*, **106**, 255-260.

[95] Challacombe, S.J., Rahman, D., Jeffery, H., Davis, S.S. & O'Hagan, D.T. (1992) Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen. *Immunology*, **76**, 164-168.

[96] Bowersock, T.L., HogenEsch, H., Suskow, M., Porter, R.E., Jackson, R., Park, H. & Park, K. (1996) Oral vaccination with alginate microsphere systems. *J.Control.Rel.*, **39** , 209-220.

[97] Rubas, W., Banerjea, A.C., Gallati, H., Speiser, P.P. & Joklik, W.K. (1990) Incorporation of the reovirus M cell attachment protein into small unilamellar vesicles: incorporation efficiency and binding capability to L929 cells in vitro. *J.Control.Rel.*, **7**, 385-395.

[98] Childers, N.K., Denys, F.R., McGee, N.F. & Michalek, S.M. (1990) Ultrastructural study of liposome uptake by M cells of rat Peyer's patch: an oral vaccine system for delivery of purified antigen. *Reg.Immunol.*, **3**, 8-16.

[99] Aramaki, Y., Tomizawa, H., Hara, T., Yachi, K., Kikuchi, H. & Tsuchiya, S. (1993) Stability of liposomes in vitro and their uptake by rat Peyer's patches following oral administration. *Pharm.Res.*, **10**, 1228-1231.

[100] Tomizawa, H., Aramaki, Y., Fujii, Y., Hara, T., Suzuki, N., Yachi, K., KikuchiH & Tsuchiya, S. (1993) Uptake of phosphatidylserine liposomes by rat Peyer's patches following intraluminal administration. *Pharm.Res.*, **10**, 549-552.

[101] Clementi, F. & Palade, G.E. (1969) Intestinal capillaries. I. Permeability to peroxidase and ferritin. *J.Cell Biol.*, **41**, 33-58.

[102] Leak, L.V. (1980) Lymphatic removal of fluids and particles in the mammalian lung. *Environmental Health Perspectives*, **35**, 55-76.

[103] Supersaxo, A., Hein, W.R. & Steffen, H. (1990) Effects of molecular weight on the lymphatic absorption of water-soluble compounds following subcutanous administration. *Pharm.Res.*, **7**, 167-169.

[104] Bocci, V., Pessina, G.P., Paulesu, L. & Nicoletti, C. (1988) The lymphatic route. VI. Distribution of recombinant interferone-alpha2 in rabbit and pig plasma and lymph. *J.Biol.Resp.Mod.*, **7**, 390-400.

[105] Hagiwara, A., Takahashi, T., Ueda, T., Iwamoto, A. & Torii, T. (1987) Activated carbon particles as anticancer drug carrier into regional lymph nodes. *Anti-Cancer Drug Design*, **1**, 313-321.

[106] Yanagawa, A., Iwayama, T., Saotome, T., Shoji, Y., Takano, K., Oka, H., Nakagawa, T. & Mizushima, Y. (1989) Selective transfer of cyclosporin to thoracic lymphatic systems by the application of lipid microspheres. *J.Microencapsulation*, **6**, 161-164.

[107] Shichiri, M., Kawamori, R., Yoshida, Y., Fukuchi, M., Shigeta, Y. & Abe, H. (1975) Short-term treatment of alloxan-diabetic rats with intrajejunal administration of water-in-oil-in-water insulin emulsions. *Diabetes*, **24**, 971-976.

[108] Oppenheim, R.C., Sperling, M.A., Gordon, L. & Patel, H.M. (1982) *Drug Dev.Ind.Pharm.*, **8** , 531-546.

[109] Kimura, T., Sato, K., Sugimoto, K., Tao, R., Murakami, T., Kurosoki, Y. & Nakayam, T. (1996) Oral administration of insulin as poly(vinyl alcohol)-gel spheres in diabetic rats. *Biol.Pharm.Bull.*, **19**, 897-900.

[110] Ziv, E., Kidron, M., Raz, I., Krausz, M., Blatt, Y., Rotman, A. & Bar-On, H. (1994) Oral administration of insulin in solid form to nnodiabetic and diabetic dogs. *J.Pharm.Sci.*, **83**, 792-794.

[111] Gwinup, G., Elias, A.N. & Domurat, E.S. (1991) Insulin and C-peptide levels following oral administration of insulin in intestinal-enzyme protected capsules. *Gen.Pharmacol.*, **22**, 243-246.

[112] Cheng, C.L., Gehrke, S.H. & Ritschel, W.A. (1994) Development of an azopolymer based colonic release capsule for delivering proteins/macromolecules. *Meth.Find.Exp.Clin.Pharmacol.*, **16**, 271-278.

[113] Weingarten, C., Moufti, A., Desjeux, J.F., Luong, T.T., Durand, G., Devissaguet, J.P. & Puisieux, F. (1981) Oral ingestion of insulin liposomes: effects of the administration route. *Life Sci.* , **28**, 2747-2752.

[114] Weingarten, C., Moufti, A., Delattre, J., Puisieux, F. & Couvreur, P. (1985) Protection of insulin from enzymatic degradation by its association to liposomes. *Int.J.Pharmaceutics*, **26**, 251-257.

[115] Choudhari, K.B., Labhasetwar, V. & Dorle, A.K. (1994) Liposomes as a carrier for oral administration of insulin: effect of formulation factors. *J.Microencapsulation*, **11**, 319-325.

[116] Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T. & Kawashima, Y. (1996) Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm.Res.*, **13** , 896-901.

[117] Dong, C. & Rogers, J.A. (1992) Poly(4-vinylpyridine)-coated liposomes: stability studies and release of acetylsalicylic acid. *J.Microencapsulation*, **9**, 437-448.

[118] Dong, C. & Rogers, J.A. (1993) Acacia-gelatin microencapsulated liposomes: preparation, stability, and release of acetylsalicylic acid. *Pharm.Res.*, **10**, 141-146.

[119] Sehgal, S. & Rogers, J.A. (1995) Polymer-coated liposomes: improved liposome stability and release of cytosine arabinoside (Ara-C). *J.Microencapsulation*, **12**, 37-47.

[120] Bangham, A.D. & Horne, R.W. (1964) Negative staining of phospholipids and their structural modification by sufrace-active agents as observed in the electron microscope. *J.Mol.Biol.*, **8**, 660-668.

[121] Chang, C.W., Barber, L., Ouyang, C., Masin, D., Bally, M.B. & Madden, T.D. (1997) Plasma clearance, biodistribution and therapeutic properties of mitoxantrone encapsulated in conventional

and sterically stabilized liposomes after intravenous administration in BDF1 mice. *Br.J.Cancer*, **75**, 169-177.

[122] Lim, H.J., Masin, D., Madden, T.D. & Bally, M.B. (1997) Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone. *J.Pharmacol.Exp.Ther.*, **281**, 566-573.

[123] Boman, N.L., Tron, V.A., Bally, M.B. & Cullis, P.R. (1996) Vincristine-induced dermal toxicity is significantly reduced when the drug is given in liposomes. *Cancer Chem.Pharmacol.*, **37**, 351-355.

[124] Bally, M.B., Nayar, R., Masin, D., Cullis, P.R. & Mayer, L.D. (1990) Studies on the myelosuppressive activity of doxorubicin entrapped in liposomes. *Cancer Chemo.Pharmacol*, **27**, 13-19.

[125] Sato, T. & Sunamoto, J. (1992) Recent aspects in the use of liposomes in biotechnology and medicine. *Prog.Lipid Res.*, **31**, 345-372.

[126] Allen, T.M., Mehra, T. & Hansen, C. (1992) Stealth liposomes: an improved sustained release system for 1-beta-D-arabinofuranosylcytosine. *Cancer Res.*, **52**, 2439

[127] Loughrey, H.C., Choi, L.S., Wong, K.F., Cullis, P.R. & Bally, M.B. (1993) Preparation of streptavidin-liposomes for use in ligand-specific targeting applications. In: *Liposome Technology: Interaction of liposomes with biological miliea*, pp. 163-178. Ed. Gregoriadis, G. CRC Press, Inc.,

[128] Mayer, L.D., Masin, D., Nayar, R., Boman, N.L. & Bally, M.B. (1995) Pharmacology of liposomal vincristine in mice bearing L1210 ascitic and B16/BL6 solid tumours. *Br.J.Cancer*, **71**, 482-488.

[129] Mayer, L.D., Nayar, R., Thies, R.L., Boman, N.L., Cullis, P.R. & Bally, M.B. (1993) Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. *Cancer Chemo.Pharmacol.*, **33**, 17-24.

[130] Lasic, D.D. (1988) Mechanism of Vesicle Formation: A Rview. *Biochem.J.*, **256**, 1-11.

[131] Seddon, J.M. (1990) Structures of the inverted hexaginal (HII) phase, and non-lamellar phase transitions of lipids. *Biochim.Biophys.Acta*, **1031**, 1-69.

[132] Kinnunen, P. K. J. and Laggner, P. Phospholipid phase transitions. (57), 109-408. 1991. Special issue of Chem. Phys. Lipids.

[133] Kinnunen, P.K.J., Koiv, A., Lehtonen, J.Y.A.R.M. & Mustonen, P. (1994) Lipid dynamics and peripheral interactions of proteins with membrane surfaces. *Chem.Phys.Lipids*, **73**, 181-207.

[134] Lasic, D.D. (1993) *Liposomes from physics to applications*. Elsevier Science B.V., Amsterdam.

[135] New, R.R.C. (1990) Introduction. In: *Liposomes, a practical approach*, pp. 1-32. Ed. New, R.R.C. Oxford University Press, New York.

[136] Finean, J.B. (1990) Interaction between cholestrol and phospholipid in hydrated bilayers. *Chem.Phys.Lipids*, **54**, 147-156.

[137] McMullen, T.P.W., Lewis, R.N.A.H. & McElhaney, R.N. (1993) Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behaviour of a homologous series of linear saturated phosphatidylcholines. *Biochemistry*, **32**, 516-522.

[138] Schullery, S.E. (1977) Permeability of iodide in multilamellar liposomes modeled by two compartments and a reservoir. *Biochim.Biophys.Acta*, **468**, 238-244

[139] Chiang, C.-M. & Weiner, N. (1987) *Int.J.Pharm.*, **40**, 143

[140] Manosori, A. & Bauer, K.H. (1989) The entrapment of a human insulin-DEAE dextran complex in different compound liposomes. *Drug Dev.Ind.Pharm.*, **15**, 2531-2546.

[141] Weiner, A.L. (1990) Lipid-based vehicles for peptide and protein drugs. Part II: manufacturing variables. *Biopharm.*, **3**, 16-21.

[142] Woodle, M.C. & Lasic, D.D. (1992) Sterically stabilized liposomes. *Biochim.Biophys.Acta*, **1113**, 171-199.

[143] Allen, T.M. & Cleland, L.G. (1980) Serum induced leakage of liposome contents. *Biochim.Biophys.Acta*, **597**, 418-426.

[144] Jones, M.W. & Nicholas, A.R. (1991) The effect of blood serum on the size and stability of phospholipid liposomes. *Biochim.Biophys.Acta*, **1065**, 145-152.

[145] Chiang, C.-M. & Weiner, N. (1987) Gastrointestinal uptake of liposomes. I. Invitro and in situ studies. *Int.J.Pharm.*, **37**, 75-85.

[146] Bader, H., Dorn, K., Hupfer, B. & Ringsdorf, H. (1985) Polymeric monolayers and liposoms as models for biomembranes. How to bridge the gap between polymer science and membrane biology? *Adv.polym.sci.*, **64**, 1-61.

[147] Ringsdorf, H., Schlarb, B. & Venzmer, J. (1988) Molecular architecture and function of polymeric oriented systems. *Angew.Chem.Int.Ed.Eng.*, **27**, 113-158.

[148] Allen, T.M. (1994) Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *TiPS*, **15**, 215-220.

[149] Longman, S.A., Tardi, P.G., Parr, M.J., Choi, L., Cullis, P.R. & Bally, M.B. (1995) Accumulation of protein-coated liposomes in an extravascular site: influence of increasing carrier circulation lifetimes. *J.Pharmacol.Exp.Ther.*, **275**, 1177-1184.

[150] Woodle, M.C. (1993) 67Gallium-labeled liposomes with prolonged circulation: preparation and potential as nuclear imaging agents. *Nucl. Med. Biol.*, **20**, 149-155.

[151] Poznansky, M.J. & Juliano, R.L. (1984) Biological approaches to the controlled delivery of drugs: a critical review. *Pharmacol.Rev.*, **36**, 277-336.

[152] Sunamoto, J. & Iwamoto, K. (1986) Protein-coated and polysaccharide-coated liposomes as drug carriers. *CRC Crit.Rev.Ther.Drug Carrier systems*, **2**, 117-136.

[153] Naisbett, B. & Woodly, J. (1994) The potential use of tomato lectin for oral drug delivery. 1. Lectin binding to rat small intestine in vitro. *Int.J.Pharm.*, **107**, 223-230.

[154] Kato, A., Arakawa, M. & Kondo, T. (1984) Preparation and stability of liposome-type artificial red blood cells stabilized with carboxymethyl chitin. *J.Microencapsulation*, **1**, 105-112.

[155] Izawa, H., Arakawa, M. & Kondo, T. (1986) Disintegration by surfactants of egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin. *Biochim.Biophys.Acta*, **855**, 243-249.

[156] Kato, A. & Kondo, T. (1987) A study of liposome-type artificial red blood cells stabilized with carboxymethyl chitin. *Polym.Sci.Technol.*, **35**, 299-310.

[157] Dong, C. & Rogers, J.A. (1991) Polymer-coated liposomes:stability and release of ASA from carboxymethyl chitin-coated liposomes. *J.Control.Rel.*, **17**, 217-224.

[158] Yeung, V.W. & Nixon, J.R. (1988) Preparation of microencapsulated liposomes. *J.Microencapsulation*, **5**, 331-337.

[159] Nixon, J.R. & Yeung, V.W. (1989) Preparation of microencapsulated liposomes, II. Systems containing nylon-gelatin and nylon-gelatin-acacia walling material. *J.Microencapsulation*, **6**, 43-52.

[160] Kibat, P.G., Igari, Y., Wheatley, M.A., Eisen, H.N. & Langer, R. (1990) Enzymatically activated microencapsulated liposomes can provide pulsatile drug release. *FASEB J.*, **4**, 2533-2539.

[161] Aliev, K.V., Ringsdorf, H. & Schlarb, B. (1984) Liposomes in a net: Spontanous polymerization of 4-vinylpyridine on acidic liposomal surfaces. *Makromol.Chem.Rapid Commun.*, **5**, 345-352.

[162] Ringsdorf, H. & Schlarb, B. (1988) Preparation and characterization of unsymetrical "liposomes in a net". *Makromol.Chem.*, **189**, 299-315.

[163] Arnold, S.C., Ferrito, M.S., Lenz, R.W. & Tirrell, D.A. (1988) pH dependent modification of phospholipid vesicle membranes by poly(carboxylic acid)s bearing pendent cholestryl esters. *Polym.Prep*, **27**, 42-43.

[164] Sunamoto, J., Sato, T., Hirota, M., Fukushima, K., Hiratani, K. & Hara, K. (1987) A newly developed immunoliposomes- an egg phosphatidyl choline liposome coated with pullulan bearing a cholesterol moiety and a IgM fragment. *Biochim.Biophys.Acta*, **898**, 323-330.

[165] Sato, T. Characterization of polysaccharide-coated liposomes and their application. 1990. Nagasaki University, Nagasaki, Japan. Ref Type: Thesis/Dissertation

[166] Ringsdorf, H., Sackmann, E., Simon, J. & Winnik, F.M. (1993) Interactions of liposomes and hydrophobically-modified poly-(N-isopropylacrylamides): an attempt to model the cytoskeleton. *Biochim.Biophys.Acta*, **1153**, 335-344.

[167] Elferink, M.G., de Wit, J.G., In 't Veld, G., Reichert, A., Driessen, A.J.R.H. & Konings, W.N. (1992) The stability and functional properties of proteoliposomes mixed with dextran derivatives bearing hydrophobic anchor groups. *Biochim.Biophys.Acta*, **1106**, 23-30.

[168] Weiner, A.L. (1994) Liposomes for protein delivery: selecting manufacture and development processes. *Immunomethods*, **4**, 201-209.

[169] Samanen, J.M. (1991) Physical biochemistry of peptide drugs: structure, properties, and stabilities of peptides compared with proteins. In: *Peptide and protein drug delivery*, pp. 137-166. Ed. Lee, V.H.L. Marcel Dekker, INC., New York.

[170] Oeswein, J.Q. & Shire, S.J. (1991) Physical biochemistry of protein drugs. In: *Peptide and protein dtug delivery* , pp. 167-202. Ed. Lee, V.H.L. Marcel Dekker, INC., New York.

[171] Ahern, T.J. & Manning, M.C. (1992) *Stability of protein pharmaceuticals. Part A: Chemical and physical pathways of protein degradation*. Plenum Press, New York.

[172] Bangham, A.D., Standish, M.M. & Watkins, J.C. (1965) Diffusion of univalant ions across the lamella of swollen phospholipids. *J.Mol.Biol.*, **13**, 238

[173] Ohsawa, T., Miura, H. & Harada, K. (1985) Evaluation of a new liposome preparation technique, the freeze thawing method, using L-asparginase as a model drug. *Chem.Pharm.Bull.*, **33**, 2916-2923.

[174] Ohsawa, T., Miura, H. & Harada, K. (1985) Improvement of encapsulation efficiency of water-soluble drugs in liposomes by freezing-thawing method. *Chem.Pharm.Bull.*, **33**, 3945-3952.

[175] Crow, J.H., Crow, I.M. & Carpenter, J.F. (1993) Preserving dry biomaterials: the water replacement hypothesis, part 1. *Biopharm.*, **6**, 28-33.

[176] Crow, J.H., Crow, L.M. & Carpenter, J.F. (1993) Preserving dry biomaterials: the water replacement hypothesis, part II. *Biopharm.*, **6**, 40-43.

[177] Higgins, J., Hodges, N.A., Olliff, C.J. & Philips, A.J. (1986) Factors influencing cryoprotective activity and drug leakage from liposomes after freezing. *J.Pharm.Pharmacol.*, **38**, 259-263.

[178] Weiner, A.L. (1989) Liposomes as drug carriers for polypeptides. *Adv.Drug Del.Rev.*, **3**, 307-341.

[179] Kirby, C. & Gregoriadis, G. (1984) Dehydration-rehydration vesicles: A simple method for high yield drug entrapment in liposomes. *Biotechnology*, **11**, 979-984.

[180] McEvoy, G.K. (1993) Insulins. In: *Drug Information*, pp. 1954-1964. Anonymous

[181] Brange, J., Langkjaer, L., Havelund, S. & Volund, A. (1991) Chemical stability of insulin. 1. Hydrolytic degradation during storage of pharmaceutical preparations. *Pharm.Res.*, **9**, 715-726.

[182] Robinson, A.B. & Rudd, C.J. (1974) Deamidation of glutaminyl and asparaginyl residues in peptides and proteins. In: *Current Topics in Cllular Regulation*, pp. 247-295. AnonymousAcademic Press, New York.

[183] Geiger, T. & Clarke, S. (1987) Deamidation, isomerization, and racemization at asparginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J.Biol.Chem.*, **262**, 785-794.

[184] Pekar, A.H. & Frank, B.H. (1972) Conformation of proinsulin. A comporison of insulin and proinsulin self association at neutral pH. *Biochemistry*, **11**, 4013-4016.

[185] Sluzky, V., Kilbanov, A.M. & Langer, R. (1992) Mechanism of insulin aggregation and stabilization in agitated aquous solutions. *Biotech.Bioeng.*, **40**, 895-903.

[186] Liu, F.-Y., Kildsig, D.O. & Mitra, A.S. (1991) Insulin aggregation in aqueous media and its effect on alpha-chymotrypsin-mediated proteolytic degradation. *Pharm.Res.*, **8**, 925-929.

[187] Randall, C.S., Malefyt, T.R. & Sternson, L.A. (1991) Approaches to the analysis of peptides. In: *Peptide and Protein Drug Delivery*, pp. 203-246. Ed. Lee, V.H.L. Marcel Dekker, INC., New York.

[188] Strickley, R.G. & Anderson, B.D. (1996) Solid-state stability of human insulin I. Mechanism and the effect of water on the kineticks of degradation in Lyophiles from pH 2-5 solutions. *Pharm.Res.*, **13**, 1142-1153.

[189] Spangler, R.S. (1990) Insulin administration via liposomes. [Review]. *Diabetes Care*, **13** , 911-922.

[190] Scott-Moncrieff, J.C., Shao, Z. & Mitra, A.K. (1994) Enhancement of intestinal insulin absorption by bile salt-fatty acid mixed micelles in dogs. *J.Pharm.Sci.*, **83**, 1465-1469.

[191] Stevenson, R.W., Parsons, J.A. & Alberti, K.G.M.M. (1981) Comparison of the methabolic responses to portal and peripheral infusions of insulin in diabetic dogs. *Metabolism*, **30**, 752

[192] Stevenson, R.W., Parsons, J.A. & Alberti, K.G.M.M. (1983) Effect of intraportal and peripheral infusions of insulin on glucose turnover and recycling in diabetic dogs. *Am.J.Physiol.*, **244**, E190-195.

[193] Stevenson, R.W., Parsons, J.A. & Alberti, K.G.M.M. (1983) Methabolic responses to intraduodonal glucose loading in insulin-infused diabetic dogs. *Am.J.Physiol.*, **245**, E200-208.

[194] Spangler, R.S. (1985) Selective insulinization of liver in conscious diabetic dogs. *Am.J.Physiol.*, **249**, E152-9.

[195] Spangler, R.S., Triebwasser, K.C. & Wilson, M.L. (1986) Effect of charge on hepatic specific nature of vesicle encapsulated insulin in conscious dogs. *Am.J.Physiol.*, **251**, E591-6.

[196] Goriya, Y., Bahoric, A., Marliss, E.B., Zinman, B. & Albisser, A.M. (1980) Blood glucose control and insulin clearance in unrestrained dogs portally infused with a portable insulin delivery system. *Diabetologia*, **19**, 452-457.

[197] Goriya, Y., Bahoric, A., Marliss, E.B., Zinman, B. & Albisser, A.M. (1982) *Am.J.Physiol.*, **242**, E335-E342

[198] Woodley, J.F. (1985) Liposomes for oral administration of drugs. [Review]. *Crit.Rev.Therap.Drug Car.Sys.*, **2**, 1-18.

[199] Okada, J., Cohen, S. & Langer, R. (1995) In vitro evaluation of polymerized liposomes as an oral drug delivery system. *Pharm.Res.*, **12**, 576-582.

[200] Sehgal, S. Formulation of pullulan-coated liposomes and release kinetics of water-soluble solutes. 1994. Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Canada. Ref Type: Thesis/Dissertation

[201] Dong, C. & Rogers, J.A. (1991) Quantitative determination of carboxymethyl chitin in polymer-coated liposomes. *J.Microencapsulation*, **8**, 153-160.

[202] Regen, S.L., Czech, B. & Singh, A. (1980) Polymerized vesicles. *J.Am.Chem.Soc.*, **102**, 6638-6640.

[203] Huggett, A.S. & Nixon, D.A. (1957) Use of glucose oxidase, peroxidase and Odianiside in determination of blood and urinary glucose. *Lancet*, **2**, 368-370.

[204] Touitou, E. & Rubinstein, A. (1986) Targeted drug delivery of insulin to rats. *Int.J.Pharm.*, **30**, 95-99.

[205] Inman, J.K. (1975) Thymus-independent antigens: The preparation of covalant, hapten-ficol conjugates. *J.Immunol.*, **114**, 704-709.

[206] Katakam, M. & Banga, A.K. (1995) Aggregation of proteins and its prevention by carbohydrate excipients: albumins and g-globulin. *J.Pharm.Pharmacol.*, **47**, 103-107.

[207] Arakawa, T., Kita, Y. & Carpenter, J.F. (1991) Protein-solvent interactions in pharmaceutical formulations. *Pharm.Res.*, **8**, 285-291.

[208] Back, J.F., Oakenfull, D. & Smith, M.B. (1979) Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry*, **18**, 5191-5196.

[209] Nishikawa, T., Akiyoshi, K. & Sunamoto, J. (1994) Supermolecular assembly between nanoparticles of hydrophobized polysaccharide and soluble protein complexation between the self aggregate of cholesterol-bearing pullulan and α-chymotrypsin. *Macromolecules*, **27**, 7654-7659.

[210] Matsuyama, K., El-Gizway, S. & Perrin, J.H. (1987) Thermodynamics of binding of aromatic amino acids to a-, b-, and g-cyclodextrins. *Drug Dev.Ind.Pharm.*, **13**, 2687-2691.

[211] Simpkins, J.W. (1991) Solubilization of ovine growth hormone with 2-hydroxy b-cyclodextrin. *J.Parenter.Sci.Technol.*, **45**, 266-269.

[212] Minetti, M., Aducci, p. & Viti, V. (1979) Interaction of neutral polysaccharides with phosphatidylcholine multilamellar liposomes. Phase transition studied by the binding of fluorescein-conjugated dextrans. *Biochemistry*, **18**, 2541-2548.

[213] Iwamoto, K. & Sunamoto, J. (1982) Liposomal membrane. XII. Adsorption of polysaccharides on liposomal membranes as monitored by fluorescence depolarization. *J.Biochem.*, **91**, 975-979.

[214] Moellerfeld, J., Prass, W., Ringsdorf, H., Hamazaki, H. & Sunamoto, J. (1986) Improved stabilty of black lipid membranes by coating with polysaccharide derivatives bearing hydrophobic anchor groups. *Biochim.Biophys.Acta*, **857**, 265-270.

[215] Lichtenberg, D., Robson, R.J. & Dennis, E.A. (1983) Solubilization of phospholipids by detergents. *Biochim.Biophys.Acta*, **737**, 285-304.

[216] Helenius, A. & Simons, K. (1975) Solubilization of membranes by detergents. *Biochim.Biophys.Acta*, **415**, 29-79.

[217] Inoue, T., Fukushima, K. & Shimozawa, R. (1990) Surfactant partition between bulk water and DPPC vesicle membrane: solid-gel vs. liquid-crystalline membrane. *Chem.Phys.Lipids*, **52**, 157-161.

[218] Urbaneja, M.-A., Alonso, A., Goni, F.M. & Partearroyo, M.A.T.M.P.S. (1990) Detegent solubilization of phospholipid vesicles: Effect of electric charge. *Biochem.J.*, **270** , 305-308.

[219] Alonso, A., Urbaneja, M.-A., Goni, F.M., Carmona, F.G., Canovas, F.G. & Gomez-Fernandez, J.C. (1987) Kinetic studies on the interaction of phosphatidylcholine liposomes with Triton X-100. *Biochim.Biophys.Acta*, **902**, 237-246.

[220] De la Maza, A., Parra, J.L. & Leal, J.S. (1992) Alteration of permeability of neutral and electronegatively charged liposomes by alkyl sulfate surfactants. *Langmuir*, **8** , 2422-2426.

[221] De la Maza, A. & Parra Juez, J.L. (1992) Disintegration of liposomes by anionic surfactants and formation of mixed micelles. *Langmuir*, **9**, 870-873.

[222] De la Maza, A. & Parra, J.L. (1995) Solubilization of unilamellar liposomes caused by quqternary ammonium surfactants. *J.Control.Rel.*, **37**, 33-42.

[223] De la Maza, A., Parra, J.L. & Garcia, M.T.R.I.L.S. (1992) Permeability changes in the phospholipid bilayer caused by nonionic surfactants. *J.Colloid Interface Sci.*, **148**, 310-316.

[224] Yotsuyanagi, T. & Mizutani, J.I.M.I.K. (1983) Kinetic aspects and mechanism of liposome disintegration in polyoxyethylene lauryl ether and sodium cholate solutions. *Biochim.Biophys.Acta*, **731**, 304-311.

[225] Racker, E. (1985) *Reconstitution of transporters, receptors and pathological states*. Academic Press, Orlando.

[226] Banuelos, S. & Muga, A. (1996) Sructural requirements for the association of native and partially folded conformations of α-lactalbumin with model membranes. *Biochemistry*, **35**, 3892-3898.

[227] New, R.R.C. (1990) Preparation of liposomes. In: *Liposomes, a practical approach*, pp. 33-104. Ed. New, R.R.C. Oxford University Press, New York.

[228] Ruf, H. and Georgalis, Y. Grell E. Dynamic laser light scattering to determine size distributions of vesicles. (172), 364-390. 1989. Academic Press Inc. Methods in Enzymology. Ref Type: Serial (Book,Monograph)

[229] Iwamoto K., Kato T., Kawahara M., Koyama N., Watanabe S., Miyake Y & Sunamoto J. (1991) Polysaccharide-coated oil droplets in oil-in-water emulsions as targetable carriers for lipophilic drugs. *J.Pharm.Sci.*, **80**, 219-224.

[230] Ikeda, T., Ledwith, A., Bamford, C.H. & Hann, R.A. (1984) Interaction of a polymer biguanide biocide with phospholipid membranes. *Biochim.Biophys.Acta*, **769**, 57-66.

[231] Boggs, J.M., Moscarello, M.A. & Papahadjopoulos, D. (1977) Phase separation of acidic and neutral phospholipids induced by human myelin basic protein. *Biochemistry*, **16** , 5420-5426.

[232] Cohen, S., Carmen Bano, M., Chow, M. & Langer, R. (1991) Lipid-alginate interactions render changes in phospholipid bilayer permeability. *Biochim.Biophys.Acta*, **1063**, 95-102.

[233] Suzuki, T., Komatsu, H. & Miyajima, K. (1996) Effects of glucose and its oligomers on the stability of freeze- dried liposomes. *Biochim.Biophys.Acta*, **1278**, 176-182.

[234] Lo, Y.L. & Rahman, Y.E. (1995) Protein location in liposomes, a drug carrier: a prediction by differential scanning calorimetry. *J.Pharm.Sci.*, **84**, 805-814.

[235] Seki, K. & Tirrell, D.A. (1984) pH-Dependent complexation of poly(acrylic acid) derivatives with phospholipid vesicle membranes. *Macromolecules*, **17**, 1692-1698.

[236] Wendlandt, W.WM. (1986) *Thermal analysis*. John Willey & sons, New York.

[237] Melling, R., Wilburn, F.W. & McIntosh, R.M. (1969) Study of thermal effects observed by differential thermal analysis. Theory and its application to influence of sample preparation on a typical DTA curve. *Anal.Chem.*, **41**, 1275

[238] Papahadjopoulos, D., Mocarello, M., Eylar, H. & Isac, T. (1975) Effects of proteins on thermotropic phase transitions of phospholipid membranes. *Biochim.Biophys.Acta*, **401**, 317-335.

[239] Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. & Thompson, T.E. (1976) A calorimetric and fluorescent probe study of the gel-liquid crystalline phase transition in small. single-lamellar dipalmitoylphosphotidylcholine vesicles. *Biochemistry*, **15**, 1393-1401.

[240] Boggs, J.M., Wood, D.D. & Moscarello, M.A. (1981) Hydrophobic and electrostatic interactions of myelin basic protein with lipid. Participation of N-terminal and C-terminal portions. *Biochemistry*, **20**, 1065-1073.

[241] Boggs, J.M., Rangaraj, G., Moscarello, M.A. & Koshy, K.M. (1985) Interaction of myelin basic proteins and polylysine with synthetic species of cerebroside sulfate. *Biochim.Biophys.Acta*, **816**, 208-220.

[242] Baszkin, A., Rosilio, V., Albrecht, G. & Sunamoto, J. (1991) Cholestryl-pullulan and cholestryl-amylopectin interactions with egg phosphatidyl choline monolayers. *J.Colloid Interface Sci.*, **145**, 502

[243] Rosilio, V., Madelmont, G., Akiyoshi, K., Sunamoto, J. & Baszkin, A. (1994) Thermal behavior of hydrated dimyristoylphosphatidylcholine / cholesteryl-pullulan mixtures. *J.Colloid Interface Sci.*, **162**, 418-424.

[244] Mouristen, O.G. & Biltonen, R.L. (1993) Protein- lipid interactions and membrane heterogenity. In: *Protein-Lipid Interactions*, pp. 1-34. Ed. Wattts, A. Elsevier, Amsterdam.

[245] Gasset, M., Onaderra, M., Martinez del Pozo, A., Schiavo, G.-P., Usobiaga, P. & Gavilanes, J.G. (1997) Effect of the antitumour protein α–sarcin on the thermotropic behaviour of acid phospholipid vesicles. *Biochim.Biophys.Acta*, **1068**, 9-16.

[246] Kraeling, M.E.K. & Ritschel, W.A. (1992) Developement of a colonic release capsule dosage form and the absorption of insulin. *Meth.Find.Exp.Clin.Pharmacol.*, **14**, 199-209.

[247] Shao, Z., Li, Y., Chermak, T. & Mitra, A.K. (1994) Cyclodextrins as mucosal absorption promoters of insulin. II. Effects of B-cyclodextrin derivatives on a-chmotryptic degradation and enteral absorption of insulin in rats. *Pharm.Res.*, **11**, 1174-1179.

[248] Li, Y. & Mitra, A.K. (1996) Effects of phospholipid chain length, concentration, charge, and vesicle size on pulmonary insulin absorption. *Pharm.Res.*, **13**, 76-79.

[249] Burvin, R., Armoni, M. & Karnieli, E. (1994) In vivo insulin action in normal and streptozocin-induced diabetic rats. *Physiol.Behav.*, **56**, 1-6.

[250] Howanitz, P.J., Howanitz, J.H. & Henry, J.B. (1991) Carbohydrates. In: *Clinical & diagnosis managed by laboratory methods*, pp. 172-187. Ed. Henry, R.R. W.B. Saunders Company, Philadelphia.

[251] Bain, O., Deville, P.J. & Mcpherson, R.A. (1991) Performance characteristics of three whole-blood glucose monitors. *Lab.Med.*, **22**, 470-474.

[252] al-Achi, A. & Greenwood, R. (1994) Human insulin absorption from the intestine in diabetic rats. *Drug Dev.Ind.Pharm.*, **20**, 2333-2339.

[253] Patel, D.G., Ritschel, W.A., Chalasani, P. & Rao, S. (1994) *Drug Dev.Ind.Pharm.*, **20**, 2333-2339.

[254] Guo, L.S.S., Radhakrishnan, R. & Redemann, C.T. (1989) Adhesion of positively-charged liposomes to mucosal tissues. *J.Liposome Res.*, **1**, 319-337.